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CORRECTIONS.

On page 538, Vol. XXXI, No. 3, September, 1917, the 5th line from the bottom, for *This, however, does disprove Scott's contention* read *This, however, does not disprove Scott's contention.*

On page 613, for

Lyxohexosamine (synthetic chondrosamine)
$\downarrow$
Lyxohexosaminic acid (synthetic)

read

Lyxohexosamine (synthetic chondrosamine)
$\uparrow$
Lyxohexosaminic acid (synthetic)

On page 619, 20th line, for *60 gm.* read *6.0 gm.*

On page 645, over the heading of the second column of the tabulation for $C_{69}H_{205}NO_{12}$ read $C_{69}H_{105}NO_{12}$.

THE FATE OF INOSITE ADMINISTERED TO DOGS.

By ISIDOR GREENWALD and MORRIS L. WEISS.

(*From the Harriman Research Laboratory, Roosevelt Hospital, New York.*)

(Received for publication, May 7, 1917.)

In a recent publication Anderson[1] has reviewed the literature on the utilization of inosite by animals and has reported the results of his own experiments. All investigators who have worked with inosite have found that it is not readily utilized by animals. After administration by mouth, a considerable amount of inosite may disappear but, after subcutaneous administration, most of it is found in the urine. The destruction of inosite given by mouth has generally been ascribed to the action of the intestinal flora. In man, Anderson found that of 0.5 gm. of inosite per kilo of body weight given by mouth, only 9 per cent was found in the urine and none in the feces. In the dog, however, with doses of 2 gm. per kilo, apparently very little was absorbed from the intestine. A large part of the inosite administered could be recovered from the feces but only a small amount was found in the urine. Ingestion of inosite did not raise the respiratory quotient and Anderson concluded that "inosite is not utilized to any extent by the dog."

At the time Anderson's experiments were published we had already been engaged with an investigation of the same subject. Our first experiments were planned to ascertain whether or not inosite was, physiologically, related to the carbohydrates. Previous work had indicated that it was not. Külz[2] and Mayer[3] had failed to observe a formation of glycogen from inosite. It is true that Mayer had found a small amount of lactic acid in the urines of rabbits receiving inosite but, since rabbits readily

[1] Anderson, R. J., *J. Biol. Chem.*, 1916, xxv, 391.

[2] Külz, E., Sitzungsber. Ges. Beförd. ges. Naturwiss., Marburg, 1876, No. 4, given in *Maly's Jahresber. Thierchem.*, 1876, vi, 45.

[3] Mayer, P., *Biochem. Z.*, 1907, ii, 393; 1908, ix, 533.

excrete lactic acid under a variety of conditions, this could not be regarded as a strong indication of a relation between inosite and carbohydrates.

In our own experiments we used phlorhizinized dogs. In these animals, although there is a normal amount of glucose in the blood, it apparently cannot be utilized. Moreover, the organism seems to form glucose from every available source. The ratio of glucose to nitrogen in the urine of fasting phlorhizinized dogs remains for days between 3.0 and 3.6 (the variation is much smaller in any one experiment) a value scarcely equalled and, in all probability, never exceeded even in the severest forms of diabetes mellitus. This ratio is assumed to represent the maximum formation of glucose from protein. If, therefore, this ratio rises after the administration of any substance, that substance may be regarded as having been converted into glucose in the organism. There are certain exceptions, such as narcotics and other toxic substances, which liberate the small amounts of glycogen which such phlorhizinized dogs still retain. In such cases the amount of glucose excreted in excess of the usual amount ("extra glucose") bears no constant relation to the amount of material administered and the effect is obtained only with the first few doses and is absent thereafter, for all the available glycogen has been excreted as glucose.[4]

At first the usual technique was employed. After the ratio of glucose to nitrogen in the urine (G:N ratio) had reached a constant level, the inosite, in aqueous solution, was injected subcutaneously. The G:N ratio rose very little. However, it was noticed that it did rise, even though very slightly, in every experiment. Unchanged inosite was also found in the urine (Tables IX and X). It was then determined to test the capacity of the normal dog to oxidize inosite. It was found that about one-half of the inosite administered was excreted unchanged (Table I). It was believed that this poor utilization might be due to the fact that the inosite was rapidly absorbed from the aqueous solution and as rapidly excreted, before the tissues had much opportunity to change it. In order to secure a more gradual absorption of the inosite, it was then given in

[4] Sansum, W. D., and Woodyatt, R. T., *J. Biol. Chem.*, 1915, xxi, 1.

suspension in cottonseed oil at intervals of 4 to 6 hours. Determinations of the ratio of total carbon to nitrogen in the urine indicated that a fairly regular excretion of inosite could be secured in this manner (Table II). An experiment with glucose and a phlorhizinized dog showed that a fairly uniform excretion of the added glucose could be secured. Also, the length of the experimental period was increased from 12 hours to at least 48 hours, in which period the inosite was administered at intervals of 4 to 6 hours.

In the normal fasting dog it was found that a considerable portion of the inosite administered could not be accounted for in the urine, either as such or by calculation from the ratio of carbon to nitrogen. Excretion into the intestine was improbable for there were usually no feces. In one experiment (Table VII) the dog was fed a constant mixture of hashed boiled beef, cracker meal, Crisco (a hydrogenated fat), bone ash, and water. The periods were marked off with carmine. Determinations of the total carbon and nitrogen in the feces were made and an attempt was made to isolate inosite from the feces of the experimental period. This was unsuccessful and the ratios of carbon to nitrogen in the feces gave no reason for believing that inosite, or any derivative thereof, was present in the feces.

A simple retention was also unlikely for in only two experiments (Tables IV and VI) was inosite found in the urine more than 12 hours after the close of the experimental period. There was a discrepancy between the amount of inosite calculated from the "extra carbon" and that actually isolated that was somewhat greater than was to be expected from the results obtained in the recovery of inosite added to urine. Among the possible derivatives of inosite were phenols and oxalic and lactic acids. The excretion of none of these was found to be increased (Tables I to IV).[5] It was thought possible that some of the inosite might be excreted, with or without previous change, in combination with sulfuric or glycuronic acids. No increase in the excretion of combined or ethereal sulfuric acid was observed.

[5] Dubin, H. (*J. Biol. Chem.*, 1916–17, xxviii, 429), has also found that the excretion of phenols in dogs is unaffected by the administration of inosite. As regards the excretion of inosite, his results are in accord with those of Anderson.[1]

There was, however, a slight, though unmistakable, increase in the excretion of glycuronic acid (Tables VI and VII). This was determined by the method of Tollens,[6] in which the glycuronic acids are first precipitated with basic lead acetate and ammonium hydroxide, the precipitate is filtered off and washed, and then treated with boiling 12 per cent hydrochloric acid, distilling off the furfurol formed. This is then precipitated as the phloroglucide.[7] There is very little evidence that the substance responsible for this increase is really glycuronic acid. The results are reported in terms of this only as a matter of convenience. That the increase was not due to the presence of inosite or of a reducing substance in the urine or to the administration of cottonseed oil was shown by suitable control experiments (Table VIII). It is very likely that the substance, whatever it may be, is identical with the dextrorotatory non-reducing substance observed by Mayer in the urine of some of the rabbits to which he administered inosite.

In the experiments with phlorhizinized dogs (Tables XI to XV) it was found that the administration of inosite in cottonseed oil was followed by a slight though unmistakable increase in the glucose: nitrogen ratio. Inosite is not a toxic or narcotic substance and the "extra glucose" can hardly be regarded as being derived from the glycogen or other carbohydrate of the body. Moreover, the amount of "extra glucose" increased as the experiment was continued, leaving very little doubt that the glucose was actually derived from the inosite administered. That it was not due to the cottonseed oil was shown by two experiments. In one of these (Table XIV) the dog received injections of cottonseed oil alone for four 12 hour periods and then, after a 24 hour interval, the inosite was administered in the usual manner. The oil alone produced only the slightest, if any, rise in the glucose: nitrogen ratio. In the inosite period, however, the usual rise was observed. In the other control experiment (Table XV) the dog received 15 cc. of cottonseed oil every 6 hours during the fore- and after-periods. This was at least

[6] Tollens, C., *Z. physiol. Chem.*, 1909, lxi, 95.

[7] In these experiments the paper was not included in the mixture subjected to distillation, thus avoiding the large correction employed by Tollens.

50 per cent more oil than was used for the suspension of the inosite. Nevertheless the glucose: nitrogen ratio rose in the usual manner with the administration of the inosite and fell thereafter.

The excretion of acetone and of β-hydroxybutyric acid was generally diminished in the experimental periods.

Determinations were made of the total carbon in the urine. From this amount were deducted the amounts of carbon present as glucose, as acetone, and as β-hydroxybutyric acid. From the residual value ("rest C"), a carbon: nitrogen ratio was calculated. From this, following the method introduced by Lusk for the calculation of the "extra glucose," the "extra carbon" and the inosite equivalent thereto were calculated. The amount of inosite actually found corresponded, as well as could be expected, with the amount calculated in this manner. In only one experiment (Table XIV) was this not true. In this experiment, particularly in one period, the amount of "extra carbon" was very high. It is possible that the excretion of some unknown constituent was very irregular, giving rise to the results obtained.

If the amount of "extra glucose" be added to the amount of inosite calculated from the "extra carbon," the sum, except in the experiment already alluded to, is almost exactly equal to the amount of inosite administered. In view of the error inherent in these determinations and calculations, the correspondence is surprisingly close. Apparently, therefore, inosite, though slowly and incompletely, is converted molecule for molecule into glucose.

EXPERIMENTAL.

The general plan of the experiments and the analytical methods were those generally employed in this laboratory.[8]

Total carbon was determined by oxidation with sulfuric acid and potassium dichromate, passing the gaseous products of oxidation through a heated combustion tube containing copper oxide and lead chromate, drying with calcium chloride, and finally absorbing the carbon dioxide in soda-lime.

Inosite was prepared from "steep water" by the method of

[8] Greenwald, I., *J. Biol. Chem.*, 1914, xviii, 115; 1916, xxv, 81.

Griffin and Nelson.[9] The isolation from the urine was accomplished by Mayer's method, crystallizing the inosite from alcohol and from acetic acid. In the experiments with phlorhizinized dogs this crystallization was repeated several times before the product was considered pure enough to weigh. The method of Meillère and Fleury[10] gave a purer product but the yield was much lower and extremely variable.

Oxalic acid was determined by Dakin's method[11] and phenols by the method of Folin and Denis.[12]

TABLE I.

Excretion of Inosite after Administration in Aqueous Solution. Normal Fasting Dogs. 12 Hour Periods.

Nitrogen.	Carbon.	C : N.	"Extra carbon."	Inosite.	Oxalic acid.	Remarks.
gm.	*gm.*		*gm.*	*gm.*	*gm.*	
0.852	0.589	0.692				
0.929	2.114	2.276	1.475	3.71		Weight 4.15 kilos. 5.81 gm. inosite in 100 cc. H_2O in 6 hourly doses in first half of period.
0.901	0.648	0.719	0.028	0.07		
0.962	0.657	0.683				
0.803	0.700	0.873			0.008	
0.996	0.859	0.864			0.020	
1.100	2.950	2.683	2.00	5.06	0.010	Weight 5 kilos. 8.05 gm. inosite in 100 cc. H_2O in 5 hourly doses in first half of period.
1.156	0.976	0.844			0.010	

The column headed inosite gives the amount of inosite calculated from the "extra carbon." The isolation of inosite from the urine was unsatisfactory because of mechanical losses, etc.

[9] Griffin, E. G., and Nelson, J. M., *J. Am. Chem. Soc.*, 1915, xxxvii, 1552. We are indebted to the Corn Products Refining Company for the supply of steep water.

[10] Meillère, G., and Fleury, P., *J. pharm. et chim.*, 1910, series 7, i, 348.

[11] Dakin, H. D., *J. Biol. Chem.*, 1907, iii, 77.

[12] Folin, O., and Denis, W., *J. Biol. Chem.*, 1915, xxii, 305.

TABLE II.

Hourly Excretion of Inosite after Administration in Suspension in Cottonseed Oil. Dog 11.

Length of period.	Nitrogen.	Carbon.	C : N.	"Extra carbon."	Inosite.	Oxalic acid.	Remarks.
hrs.	*gm.*	*gm.*		*gm.*	*gm.*	*gm.*	
12	0.963	0.873	0.906			0.011	
12	0.826	0.728	0.882				
2	0.175	0.259	1.49	0.102	0.256		Weight 8 kilos. 4 gm. inosite in 10 cc. cottonseed oil.
2	0.183	0.376	2.06	0.212	0.533		
2	0.190	0.658	3.46	0.487	1.223		4 gm. inosite in 10 cc. cottonseed oil.
2	0.192	0.791	4.03	0.600	1.508		
2	0.175	0.547	3.14	0.390	0.981		
2	0.144	0.316	2.19	0.186	0.468		
2	0.169	0.261	1.54	0.108	0.272		
Total........	1.208	3.208		2.085	5.241		
Composite...	1.228	3.267		2.172	5.461	0.005	
10	1.180	1.201	1.017	0.138	0.347	0.008	
12	1.347	1.207	0.896			0.009	

A composite of the 2 hour urines was prepared and analyzed, with the results given above. The values for inosite are those calculated from the amount of "extra carbon." 4.4 gm. of inosite were isolated from the composite urine.

TABLE III.

Excretion of Inosite after Administration in Oil for Four 12 Hour Periods. Dog 17, Normal, Fasting, Weight 9 Kilos.

Nitrogen.	Carbon.	C : N.	"Extra carbon."	Oxalic acid.	Inosite.		
					Calculated.	Found.	Administered.
gm.	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
2.096	1.571	0.750		0.0045			
1.591	1.371	0.861		0.0030			
1.415	1.579	1.116	0.306	0.0023	0.769	0.50	4.85
1.639	2.874	1.754	1.400	0.0034	3.520	2.70	6.00
1.939	2.944	1.519	1.200	0.0020	3.017	3.05	6.00
2.450	3.710	1.514	1.505	0.0017	3.780	3.10	6.00
2.205	2.210	1.000	0.221	0.0045	0.554	0.34	
1.575	1.550	0.987	0.137	0.0023	0.350	0.00	
1.621	1.506	0.929					
1.309	1.230	0.940					
Total.....	....	....	....	....	11.99	9.69	22.85

TABLE IV.

Excretion of Inosite after Administration in Oil for Four 12 Hour Periods. Dog 21, Normal, Fasting, Weight 12 Kilos.

Nitrogen.	Carbon.	C : N.	"Extra carbon."	Phenols.	Inosite.		
					Calculated.	Found.	Administered.
gm.	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1.156	0.981	0.848		0.045			
1.034	0.965	0.933		0.049			
0.995	2.289	2.301	1.39	0.047	3.50	2.56	9.2
0.932	3.600	3.864	2.76	0.058	6.94	5.33	8.0
1.448	3.926	2.711	2.62	0.066	6.59	5.40	9.0
1.422	4.023	2.829	2.74	0.066	6.90	5.10	8.0
1.311	1.802	1.375	0.62	0.054	1.56	1.30	
1.259	1.225	0.973	0.09	0.057	0.23	0.35	
1.280	1.354	1.058	?*	0.068	?*	0.13	
1.144	1.023	0.895		0.067			
Total.....	....	....	....	....	25.72	20.17	34.2

*The urine was contaminated with blood from a wound. The C : N ratio is probably too high and the "extra carbon" and inosite have therefore not been calculated.

TABLE V.

Excretion of Inosite after Administration in Oil for Four 12 Hour Periods. Dog 25, Normal, Fasting, Weight 20 Kilos.

Nitrogen.	Carbon.	C : N	"Extra carbon."	Inosite.		
				Calculated.	Found.	Administered.
gm.	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*
2.360	2.113	0.895				
2.565	2.240	0.873				
2.888	3.100	1.073	0.965	2.43	1.70	8.46
3.637	3.947	1.085	1.258	3.16	2.50	8.00
5.057	5.103	1.002	1.330	3.34	3.37	8.00
5.642	7.062	1.252	2.894	7.28	6.77	8.00
4.970	3.802	0.765				
4.431	3.103	0.700				
2.528*	1.902	0.752				
Total.....	....	....	....	16.21	14.34	32.46

*10 hour period.

The "extra carbon" and inosite have been calculated upon the basis of a basal C : N ratio of 0.739, the average of the last three periods. This gives the maximal values for "extra carbon" and inosite.

TABLE VI.

Excretion of Inosite after Administration in Oil for Four Successive 12 Hour Periods. Dog 25, Weight 19 Kilos.

Nitrogen.	Carbon	C : N	"Extra carbon."	Inosite. Calculated.	Inosite. Found.	Inosite. Administered.	Sulfur. Inorganic.	Sulfur. Combined SO_4.	Glycuronic lactone	Period.
gm.	gm.		gm.	gm.	gm.	gm.	gm.	gm.	gm.	hrs.
2.052	1.502	0.732					0.084	0.016	0.125	10.5
2.336	1.781	0.763					0.097	0.018	0.148	12
2.622	3.676	1.402	1.72	4.32	3.84	12.4	0.091	0.017	0.189	12
2.622	4.528	1.727	2.57	6.46	5.99	10.0	0.092	0.016	0.176	12
3.926	5.829	1.487	3.29	8.28	6.94	12.0	0.131	0.016	0.250	12
4.075	6.451	1.583	3.81	9.58	7.67	10.0	0.118	0.012	0.232	12
4.550	3.857	0.848	0.91	2.29	1.74		0.164	0.015	0.211	12
4.774	3.401	0.712	0.31	0.77	0.31		0.163	0.014	0.162	13
3.474	2.248	0.647							0.165	10
4.301	2.789	0.649								14.5
Total..	......	......	12.61	31.70	26.49	44.4				

The basal C : N ratio has been taken as 0.747 (the average of the ratios in the fore-periods) for the first two experimental periods and as 0.648 (the average of the ratios in the last two periods) for the others.

TABLE VII.

Excretion of Inosite after Administration in Oil for Four 24 Hour Periods. Dog 25, Weight 19 Kilos. Fed Daily.

Food:	gm.
Boiled beef	190
Cracker meal	76
Crisco	57
Bone ash	19
Water	760 cc.

Nitrogen.	Carbon.	C : N.	"Extra carbon"	Inosite. Calculated.	Inosite. Found.	Inosite. Administered.	Sulfur. Inorganic.	Sulfur. Combined SO_4.	Glycuronic lactone.	Feces. Carbon.	Feces. Nitrogen.	Feces. C : N.
gm.	gm.		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	
11.10	7.18	0.647					0.452	0.076	0.338			
9.75	6.30	0.646					0.358	0.078	0.320	20.7	1.61	12.9
9.22	9.92	1.076	3.93	9.87	8.32	20.9	0.323	0.061	0.489			
9.87	12.23	1.239	5.81	14.61	13.89	22.0	0.286	0.051	0.464			
10.51	12.68	1.207	5.85	14.72	13.87	22.0	0.379	0.060	0.412			
11.87	11.79	0.993	4.07	10.24	8.01	22.0	0.443	0.045	0.522	34.4	3.38	10.2
10.49	6.87	0.655					0.412	0.049	0.280			
10.47	6.82	0.652					0.441	0.049	0.309	16.6	1.73	9.6
Total..	.....	.....	.....	49.44	44.09	86.9						

TABLE VIII.

Effect of Subcutaneous Administration of Cottonseed Oil on the Excretion of Glycuronic Acid. Dog 25, Weight 19 Kilos. Fed Daily. Periods 24 Hours Each.

Food:

	gm.
Boiled beef	190
Cracker meal	76
Crisco	57
Bone ash	19
Water	760 cc.

Nitrogen.	Glycuronic lactone.	Cottonseed oil.
gm.	*gm.*	*cc.*
11.56	0.300	
10.97	0.348	
10.25	0.325	4 × 20
10.57	0.349	4 × 20
9.90	0.308	3 × 20
10.31	0.349	4 × 20
Lost.		
9.97	0.355	
9.01	0.308	

TABLE IX.

Inosite Administered in Aqueous Solution to a Phlorhizinized Dog, Weight 9.5 Kilos. Periods 12 Hours Each.

Nitrogen.	Glucose.		G : N.	"Extra glucose."	Acetone.	β-Hydroxybutyric acid.	Carbon.				Inosite.		
	Benedict.	Polariscope.					Total.	"Rest."	C : N.	"Extra."	Calculated.	Found.	Doses administered.
gm.	*gm.*	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
10.08	32.5	30.4	3.22		0.091	0.441	19.39	6.21	0.617				
8.95	29.1	28.1	3.25		0.091	0.573	17.02	5.10	0.572				
6.92	24.9	24.8	3.61	1.94	0.065	0.482	19.64	9.46	1.365	5.04	12.7	8.6	14.4
7.18	24.2	23.6	3.37		0.077	0.448	14.76	4.87	0.678				
5.83	20.4		3.49		0.094	0.629	12.44	4.00	0.686				

TABLE X.

Inosite Administered in Aqueous Solution to a Phlorhizinized Dog, Weight 12 Kilos. Periods 12 Hours Each.

Nitrogen.	Glucose.		G : N.	"Extra glucose."	Acetone.	β-Hydroxybutyric acid.	Carbon.				Inosite.		
	Benedict.	Polariscope.					Total.	"Rest."	C : N.	"Extra."	Calculated.	Found.	Administered.
gm.	gm.	gm.		gm.	gm.	gm.	gm.	gm.		gm.	gm.	gm.	gm.
5.483*	18.57	18.6	3.39		0.039	0.120	11.15	3.68	0.671				
6.205	19.60	19.6	3.16		0.060	0.234	12.52	4.58	0.738				
5.915	22.36	22.8	3.78	2.49	0.049	0.215	18.01	8.98	1.519	4.62	11.6	8	12.7
5.817	19.94	19.9	3.43		0.139	0.793	12.67	4.29	0.738				
6.355	22.01	21.8	3.46		0.099	0.430	13.65	4.64	0.730				

*11 hour period.

TABLE XI.

Inosite Administered in Suspension in Oil to a Phlorhizinized Dog, Weight 20.6 Kilos. Periods 12 Hours Each.

Nitrogen.	Glucose.		G : N.	"Extra glucose."	Acetone.	β-Hydroxybutyric acid.	Carbon.				Inosite.		
	Benedict.	Polariscope.					Total.	"Rest."	C : N.	"Extra."	Calculated.	Found.	Administered.
gm.	gm.	gm.		gm.	gm.	gm.	gm.	gm.		gm.	gm.	gm.	gm.
4.635*	16.34	15.9	3.53		0.197	2.37	12.09	4.37	0.943				
7.026	25.62	24.5	3.65		0.173	3.08	17.63	5.97	0.843				
6.852	27.10	26.3	3.95	2.12	0.236	2.27	19.75	7.78	1.136	2.34	5.88	5.12	10.5
7.183	26.09	25.4	3.63		0.319	3.14	18.13	6.10	0.849	0.39	0.98	0.58	
7.150	25.66		3.59		0.194	2.72	17.10	5.53	0.791				
4.576**	14.69		3.21		0.084	0.62	9.83	3.65	0.798				
Total...	.....	.....	.....	.....	.....	.....	.....	.....	.....	2.73	6.86	5.70	

*8 hour period.

**6 hour period.

The "rest" carbon and the inosite have been calculated upon the basis of a basal C : N ratio of 0.795, which gives the maximal values for "extra" carbon and inosite.

Inosite Administered in Suspension in Oil to a Phlorhizinized Dog, for Four Successive 12 Hour Periods.

Nitrogen.	Glucose.		G : N.	"Extra glucose."	Acetone.	β-Hydroxybutyric acid.	Carbon.				Inosite.		
	Benedict.	Polariscope.					Total.	"Rest."	C : N.	"Extra."	Calculated.	Found.	Administered.
gm.	*gm.*	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*
7.104	24.57	24.7	3.46										
7.068	26.00	26.4	3.68		0.451	4.26	16.74	4.15	0.587				
7.244	24.65	24.7	3.40		0.525	4.57	17.92	5.68	0.784				
7.278	28.70	29.1	3.77	0.51	0.184	4.72	22.24	8.53	1.172	2.71	6.81	4.15	10.45
6.937	27.62	28.6	3.98	1.94	0.169	2.66	20.22	7.90	1.139	2.35	5.88	3.81	10.45
6.352	25.39	24.2	4.00	3.68	0.079	0.82	18.37	7.84	1.234	2.76	6.93	4.54	12.45
5.820	24.92	24.7	4.28	3.84	0.060	0.51	18.52	8.23	1.414	3.57	8.99	6.76	10.45
5.205	22.18	21.9	4.36	0.85	0.013	0.07	14.75	5.89	1.132	1.73	4.34	2.10	
3.854	14.89	14.9	3.87		0.007	0.03	9.22	3.18	0.825				
3.226	11.77	11.2	3.74				6.98	2.28	0.707				
1.651	6.18		3.75										
Total..	.....	....	....	10.82						13.12	32.95	21.36	43.80

The calculations have been made upon the basis of basal ratios of 3.70 and 0.800 for G : N and C : N, respectively. It is believed that these ratios give minimal values for "extra glucose" and maximal values for inosite.

TABLE XIII.

Inosite Administered in Suspension in Oil to a Phlorhizinized Dog, Weight 15 Kilos, for Four Successive 12 Hour Periods.

Nitrogen.	Glucose.		G : N.	"Extra glucose."	Acetone.	β-Hydroxybutyric acid.	Carbon.				Inosite.		
	Benedict.	Polariscope.					Total.	"Rest."	C : N.	"Extra."	Calculated.	Found.	Administered.
gm.	*gm.*	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*
3.851*	12.81	12.3	3.33		0.044	0.167	7.11	1.91	0.495				
7.658	24.40	23.3	3.19		0.120	0.463	14.01	4.02	0.525				
8.395	27.54	27.3	3.28	0.92	0.128	0.829	19.13	7.72	0.920	1.01	2.53	2.62	10.2
8.160	28.52	27.2	3.49	2.61	0.133	0.544	21.11	9.44	1.157	2.10	5.27	6.63	12.4
7.788	27.46	26.5	3.53	2.80	0.087	0.339	21.31	10.18	1.307	3.95	9.93	7.55	12.1
7.359	25.54	26.3	3.47	2.21	0.102	0.308	18.96	8.60	1.169	2.72	6.83	6.53	9.0
7.349	25.23	24.9	3.43	1.91	0.092	0.293	17.04	6.66	0.906	0.78	1.96	2.18	
7.148	22.61	22.7	3.16		0.097	0.292	15.43	6.24	0.873	0.52	1.31	0.00	
6.315	19.95	20.5	3.16		0.051	0.222	12.94	4.87	0.772				
5.708	19.03		3.33		0.054	0.156	12.56	4.89	0.857				
Total..	.....	....	....	10.45						11.08	27.83	25.51	43.7

*6 hour period.

The "extra glucose" has been calculated upon the basis of a dominant ratio of 3.17. The wide variations in the value of the C : N ratio made the selection of a dominant ratio difficult. The value finally selected and used was 0.80.

TABLE XIV.

Effect of Administration of Cottonseed Oil Alone, and of Inosite in Oil, to a Phlorhizinized Dog, Weight 11.6 Kilos. Periods 12 Hours Each.

Nitrogen.	Glucose. Benedict.	Glucose. Polariscope.	G : N.	"Extra glucose."	Carbon. Total.	Carbon. "Rest."	Carbon. C : N.	Carbon. "Extra."	Cottonseed oil.		
gm	gm.	gm.		gm.	gm.	gm.		gm.	cc.		
9.559	32.36		3.39								
4.812*	16.74		3.48								
4.931*	17.29		3.51								
10.02	36.31		3.62						40		
9.561	35.04		3.67						60		
9.504	33.48		3.52						40		
8.857	30.19		3.41						60		
									Inosite.		
									Calculated.	Found	Administered.
									gm.	gm.	gm.
8.478	29.72		3.51								
3.950*	13.44	13.0	3.40		8.68	3.34	0.844				
4.197*	14.22	14.5	3.30		8.92	3.26	0.778				
7.774	30.30	29.1	3.90	6.10	20.17	8.12	1.045	2.00	5.02	3.18	7.88
7.723	28.65	28.7	3.71	4.09	20.49	9.10	1.178	3.01	7.57	3.36	12.0
6.656	26.85	26.4	4.04	5.72	18.36	7.68	1.154	2.43	6.12	3.12	10.0
5.793	21.98	21.1	3.80	3.59	16.92	8.18	1.412	3.62	9.09(?)**	2.83	12.0
5.124	18.63	17.6	3.64	2.35	12.72	5.31	1.061	1.39	3.52	0.18	
2.743*	8.42		3.07		5.51	2.16	0.784				
3.128*	9.21		2.94		5.99	2.33	0.745				
5.946	17.33		2.91								
Total..	.....	.....	.....	21.85				12.45	31.32	12.67	41.88

*6 hour period.

**Note the unusual discrepancy between amount of inosite calculated from "extra" carbon and that actually found.

TABLE XV.

Inosite, Suspended in Cottonseed Oil, Administered to a Phlorhizinized Dog Receiving Oil throughout the Experiment. Dog 29, Weight 22 Kilos.

Nitrogen.	Glucose.		G : N.	"Extra glucose."	Carbon.				Inosite.		
	Benedict.	Polariscope.			Total.	"Rest."	C : N.	"Extra."	Calculated.	Found.	Administered.
gm.	gm.	gm.		gm.	gm.	gm.		gm.	gm.	gm.	gm.
6.010	19.39		3.22								
6.222	19.53	19.1	3.14								
12.06	35.86	35.3	2.97		23.14	8.80	0.736				
11.36	36.74	36.7	3.23	1.64	24.18	9.56	0.842	1.10	2.77	1.80	9.7
11.09	38.00	37.9	3.43	3.72	26.39	11.29	1.002	2.85	7.17	6.43	10.0
10.85	38.59	38.1	3.56	5.07	26.42	11.07	1.020	2.98	7.50	6.54	10.0
10.35	37.95	37.6	3.67	5.98	25.73	10.64	1.028	2.93	7.36	5.51	10.0
9.65	33.77	32.8	3.50	3.95	20.75	7.29	0.755				
9.57	30.65	27.8	3.20		19.78	7.59	0.793				
Total.	......	......	......	20.36				9.86	24.80	20.28	39.7

THE CITRIC ACID FERMENTATION OF ASPERGILLUS NIGER.*

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Plates 1 and 2.

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INTRODUCTION.

The citric acid fermentation induced by certain fungi has been elaborately studied, especially by Wehmer.[1] His work on this subject and also on the oxalic acid fermentation is familiar to all students of fungi. Wehmer[2] believed that the production of citric acid in more than mere traces was characteristic of the group of fungi to which he gave the generic name *Citromyces* and that the oxalic acid fermentation was characteristic of *Aspergillus niger*. This seems to have been accepted by all the other workers who have investigated either the citric or oxalic acid fermentation. Martin,[3] in a very recent study of the citric acid fermentation, discarded all cultures of *Aspergilli* with the assumption that their fermentative action was well known and that they did not produce citric acid.

It has been noted[4] that many cultures of *Aspergillus niger* produced citric acid. Although the literature on the chemical activity of *Aspergillus niger* is voluminous, only one reference has been found relative to citric acid production by this group

* Published with the permission of the Secretary of Agriculture.

[1] Wehmer, C., Beitr. zur Kenntnis einheimscher Pilze, Hannover, 1893, No. 1.

[2] Wehmer, in Lafar, F., Handb. technischen Mykologie, Jena, 2nd edition, 1905–07, iv, 242.

[3] Martin, J. A., *Am. J. Pharm.*, 1916, lxxxviii, 337.

[4] Thom, C., and Currie, J. N., *J. Agric. Research*, 1916, vii, 1.

of fungi. In 1913 Zahorski[5] was granted a patent in the United States on a method for producing citric acid by fermenting sugar solutions with *Sterigmatocystis nigra.* This is one of the many names that has been used to designate fungi of the black *Aspergillus* group.[4] Zahorski, however, states that *Sterigmatocystis* differs distinctly from *Aspergillus.*

The writer at first supposed that Zahorski had worked with some very unusual culture of *Aspergillus niger.* This impression was probably wrong, for any one of about twenty cultures studied under certain conditions produced citric acid in abundance. In fact almost any culture of *Aspergillus niger* upon concentrated sugar solutions will produce much more citric acid than oxalic acid. For conducting the citric acid fermentation a well selected culture of *Aspergillus niger* is far superior to any culture resembling Wehmer's *Citromyces* with which the writer has ever worked.

In the beginning it was hoped that *Aspergillus niger* cultures could be divided into two general groups, one of which produced citric acid and the other oxalic acid. This would lend some aid to the problem of classifying this puzzling group of black *Aspergilli.* In this respect the data are disappointing. No cultures produced citric acid only under all conditions or oxalic acid only under all conditions.

Many of the workers who have studied the citric acid fermentation performed only a few experiments without being guided by a fundamental knowledge of the metabolism of fungi or of the conditions favorable to the reaction with which they were concerned. Experiments conducted in this way are not likely to make a very definite contribution to any problem. In this paper three fundamental factors with regard to *Aspergillus niger* have been considered: (1) The inorganic salt requirements; (2) the general equation of metabolism; and (3) the reaction of the medium.

Few concise statements can be made concerning the metabolism of an organism capable of producing such a variety of chemical transformations as *Aspergillus niger.* What is true for one set of conditions may not be true for another set of conditions differing ever so little. Much of the preliminary work which served no other useful end than to inform the experimenter will not be

[5] Zahorski, B., U. S. Patent No. 1,066,358, July 1, 1913.

described in detail, although general conclusions from such preliminary work may be related. Results were sometimes obtained which could not be duplicated. Such results have not been included without comment to this effect.

Methods Employed.

The cultures were selected from those used in the previous study and are designated by the same numbers, with the omission of the first two digits.

The chemical reactions must proceed in the mycelium. This floats on the surface of the substrate but wrinkles in such a manner that it presents an enormous surface of contact. These wrinkled structures often project 5 to 6 cm. into the substrate. This peculiarity of growth enables the mold to exhaust a deep substrate much more rapidly than if it depended on diffusion alone. Nevertheless the ratio of the surface to the volume of the media must be uniform in order to obtain results that have comparative values. A volume of 50 cc. of media contained in a 200 cc. Erlenmeyer flask was used in nearly all of the experiments here reported. The cultures were grown at 28°C.

To determine the quantitative relations between the products formed and the sugar consumed, the medium was drained off and the mycelium repeatedly washed. The medium and washings were combined and made up to a definite volume. Separate portions of this solution were then taken for the estimation of oxalic acid, citric acid, and sugar.

Oxalic acid was in all cases estimated by double precipitation as calcium oxalate and titration with standard permanganate. The sugar was estimated by reduction with Fehling's solution and calculated from the reducing factor of invert sugar. The citric acid was estimated either by the method of Pratt[6] or the method of Kunz.[7] While either method will give fairly satisfactory results if used with discretion and patience, a really convenient and accurate method for estimating citric acid is still wanting.

Carbon dioxide was determined by drawing a gentle current of

[6] Pratt, D. S., *U. S. Dept. Agric., Bureau of Chemistry, Circ. 88*, 1912.
[7] Kunz, R., *Arch. chem. Mikros.*, 1914, vii, 285.

carbon-dioxide-free air through the flasks containing the growing mold and absorbing the carbon dioxide produced in caustic potash.

Diligent search was made for other organic acids, especially malic and tartaric. These were never found and in all probability are never formed at all. The total acidity is nearly exactly accounted for by the sum of the oxalic and citric acids regardless of the proportion in which they occur.

Both citric and oxalic acids have repeatedly been isolated and identified. The oxalic acid can be recovered directly from the fermented liquors by evaporation and crystallization. Citric acid because of its very great solubility has been recovered only through the calcium salt. Several pounds of calcium citrate have been prepared which by analysis corresponds to the formula $Ca_3(C_6H_5O_7)_2 4H_2O$ and in no wise differs from calcium citrate prepared from the citrus fruits. The citric acid prepared from this calcium citrate by decomposition with sulfuric acid has sometimes crystallized out anhydrous and sometimes with one molecule of water.

There is some discussion in the literature on the isomerism of citric acid. Witter[8] claimed that an anhydrous isomeric citric acid was obtained by recrystallizing a sample of ordinary citric acid which had been dehydrated at 130°C. His conclusion was based on chemical data. On the other hand, Meyer[9] concluded from physicochemical data that the acids were identical. The anhydrous acid was the more stable form at high temperatures and the hydrated acid the more stable form at low temperatures. The conclusions of Meyer are in all likelihood correct.

Mineral Requirements of Fungi.

Earlier workers with fungi used very complex culture media. Raulin's[10] fluid which is still much used by mycologists is a good illustration of this type of medium. The composition as given by Raulin is as follows:

[8] Witter, H., *Ber. chem. Ges.*, 1892, xxv, 1159.
[9] Meyer, J., *Ber. chem. Ges.*, 1903, xxxvi, 3599.
[10] Raulin, J., *Ann. Sc. nat.* (*sér. 5, Botanique*), 1869, ii, 224.

	gm.
Distilled water	1,500 cc.
Cane sugar	70
Tartaric acid	4
Ammonium tartrate	4
Ammonium phosphate	0.6
Potassium carbonate	0.6
Magnesium carbonate	0.4
Ammonium sulfate	0.25
Zinc sulfate	0.07
Ferrous sulfate	0.07
Potassium silicate	0.07

Although Raulin conducted a very elaborate research on the mineral requirements of fungi, he made his formula much more complex than was really necessary. This was shown by the later work of Nägeli,[11] Molisch,[12] and Benecke.[13] The combined work of these three investigators showed that after a mold had been supplied with available sources of carbon and nitrogen, four elements only were necessary for growth—potassium, magnesium, phosphorus, and sulfur. No one of these four elements can be replaced by any element of the same chemical group. Molisch thought iron was necessary for the complete development from spore to spore. Benecke tried to settle this question but acknowledged that his results were inconclusive. In some cases when no iron salts were added to the media there was no sporification while in other cases there was an abundant development of spores.

After observing a very large number of cultures of *Aspergillus niger* upon media to which no iron salts had been added the writer is of the opinion that iron is not at all necessary for the development of spores. Indeed in some media iron salts do not even stimulate the development of mycelium or accelerate the rate of metabolism in any way. The only practicable forms in which nitrogen can be offered in inorganic combination are ammonium salts and nitrates. The addition of iron salts to media containing nitrates always accelerates the rate of metabolism and increases the weight of mycelium, while if nitrogen be supplied by ammonium salts, of which the ammonium phosphates serve best,

[11] Nägeli, C., *Sitzungsber. Akad. Wissensch. München*, 1880, x, 340.
[12] Molisch, H., *Sitzungsber. Akad. Wissensch. Wien*, 1894, ciii, 554.
[13] Benecke, W., *Pringsheim's Jahrb. wissench. Botanik*, 1895, xxviii, 487.

the addition of iron is entirely without effect. This suggests that some definite chemical reaction involved in the utilization of nitrates is accelerated in the presence of iron. Data in support of this are offered in another section of this paper.

Nearly all media used by mycologists contain at least a "trace of iron." The contention is frequently made that this "trace" which is necessary can be obtained from the walls of the glass containers. In order to settle this point a medium was prepared of the following composition:

	gm.
Distilled water	1,000 cc.
Cane sugar	30
Ammonium dihydrogen phosphate	2
Potassium chloride	0.2
Magnesium sulfate	0.2

The water used in making up the medium and for recrystallizing the salts was doubly distilled and received in flasks lined with a pure high melting paraffin. All the salts were recrystallized three times in porcelain dishes and finally twice in a platinum dish. The cane sugar was crystallized five times from redistilled alcohol and acetone, and finally twice from alcohol in a platinum dish. To what extent this method has been used to purify saccharose is not known to the writer. Ethyl alcohol of 90 per cent strength is saturated at the boiling point with saccharose. When the solution has cooled, acetone is added until an abundant precipitate of saccharose falls out.

The medium was sterilized in a platinum dish covered with tin foil and, when cool, poured into sterile paraffined flasks. With all of these precautions to assure the absence of iron, *Aspergillus niger* spored just as abundantly as on a medium of the same composition to which iron salts were added. Transfers to this iron-free medium from cultures already grown upon it likewise showed no impairment of ability to produce spores (Fig. 1).

The same cultures are seen in Fig. 2 grown upon a medium containing iron, but failing in all but two cases to form more than a few scattered patches of spores. This medium had the following composition per 1,000 cc.

	gm.
Saccharose	150
NH_4NO_3	2.5
KH_2PO_4	1.0
$MgSO_4.7H_2O$	0.2
$FeSO_4.7H_2O$	0.01
HCl	10 cc. N/5

It might be added here that the iron-free medium described above plus 1.5 per cent of agar has been used for some time to carry about 60 stock cultures of *Aspergilli* and *Penicillia*. Nearly every culture grows more luxuriantly on this simple three salt medium than on many of the more complex media proposed in the botanical literature. It supplies the required nutrients in about the correct proportion, has a reaction favorable to most fungi, and remains clear when sterilized.

While considering the subject of the mineral requirements of fungi, a further word might be included concerning the action of stimulants. Raulin[10] noted that the addition of a little zinc sulfate to a medium stimulated growth. Many other substances will also stimulate growth. One might suppose that the addition of some of these stimulants would accelerate the rate of the citric acid fermentation and perhaps even increase the yield of acid. On this point the writer's experience agrees in general with the conclusion of Watterson[14] that the action of such stimulants enables the fungus to transform more of the food material into its own body substance and less into waste products. In accord with this principle it is clear that the highest yield of fermentation by-products will be had when the development of mycelium is restricted and not when stimulated.

The General Equation of Metabolism of Aspergillus niger.

The general equation of metabolism of *Aspergillus niger* may be written in the following form:

Carbohydrate→citric acid→oxalic acid→carbon dioxide→mycelium.

These four products are nearly always present. Although their proportion may vary widely with the culture employed and the conditions of growth, their sum will account for approximately 95 per cent of the consumed carbohydrate.

[14] Watterson, A., *Bull. Torrey Bot. Club*, 1904, xxxi, 291.

We commonly think of respiration as an oxidation which proceeds with the consumption of oxygen and the elimination of carbon dioxide. Throughout the plant kingdom there are numerous instances where this respiratory process stops short of carbon dioxide, and results in the formation of the common organic acids, tartaric, oxalic, citric, malic, and succinic. All of

TABLE I.

Composition of Media in Gm. per 1,000 Cc.

No.	Saccharose.	Nitrogen.		KH_2PO_4	KCl	$MgSO_4 \cdot 7H_2O$	$FeSO_4 \cdot 7H_2O$
1	50	$NaNO_3$	1.5	1.0		0.2	
2	50	"	2.0	1.0		0.2	
3	50	"	3.0	1.0	0.5	0.5	0.01
4	100	"	3.0	1.0	0.5	0.5	0.01
5	150	NH_4NO_3	2.5	1.0		0.25	
6	150	"	2.5	1.0		0.25	
7	50	$NH_4H_2PO_4$	2.0		0.2	0.4	
8	50	"	2.0		0.2	0.4	
9	50	"	2.0		0.2	0.2	0.01
10	50	"	2.0		0.2	0.2	
11	50	"	2.0		1.0	0.2	0.01
12	50	"	2.0		1.0	0.2	
13	50	"	2.0		2.0	0.2	0.01
14	50	"	2.0		2.0	0.2	
15	50	Asparagine.	2.0		0.2	0.2	0.01
16	50	"	2.0		0.2	0.2	
17	50	$NaNO_3$	2.5	1.0		0.2	
18	50	"	2.5	1.0		0.2	
19	50	NH_4NO_3	1.2	1.0		0.2	0.01
20	50	"	1.2	1.0		0.2	

these acids have been anticipated as products of the action of fungi but only oxalic and citric have ever been definitely identified. The fermentation of a sugar by *Aspergillus niger* may be considered as an oxidation proceeding in three stages, and producing citric acid, oxalic acid, and carbon dioxide. This reaction can be controlled to a very considerable extent. The measure of control is the measure of success in conducting the oxalic acid or the citric acid fermentation. Generally speaking, the conditions most favorable for a high yield of the end-products, carbon dioxide and mycelium, are least favorable for the formation of the intermediate products, citric and oxalic acids.

The equation has been written in this order because *Aspergillus niger* will readily form oxalic acid when given only citric acid as a source of carbon. Carbon dioxide always accompanies the development of mycelium. In fact it is given off in greatest quantities between the 2nd and 5th days when the growth of mycelium is most rapid.

There is a common tendency among investigators to study

TABLE II.

Products of Metabolism in Gm. of Aspergillus niger upon 50 Cc. of Media of the Composition Given in Table I.

No.	Culture.	Age.	Saccharose consumed.	Carbon dioxide.	Oxalic acid.	Citric acid.	Mycelium.
		days					
1	142	8	1.797	1.2542	0.4914	0.1220	0.372
2	142	9	2.126	1.3638	0.6048	0.0439	0.520
3	142	8	2.268	1.5514	0.6401	0.0000	0.641
4	142	8	3.468	1.9757	0.4133	0.5812	0.775
5	28.7	8	6.632	1.2061	0.0945	3.5000	0.969
6	28.7	8	5.926	1.4356	0.0158	3.2906	0.985
7	142	8	1.686	0.7809	0.5353	0.2907	0.525
8	69.4	8	1.707	0.6348	0.2041	0.5801	0.481
9	28.7	7	1.353	0.6345	0.0869	0.3139	0.417
10	28.7	7	1.530	0.6743	0.1310	0.5012	0.380
11	28.7	7	1.558	0.7288	Trace.	0.4221	0.507
12	28.7	7	1.582	0.7368	"	0.4475	0.498
13	28.7	7	1.482	0.7835	"	0.3342	0.475
14	28.7	7	1.410	0.7905	"	0.3106	0.477
15	28.7	7	*	0.5254	0.1739	0.4116	*
16	28.7	7	*	0.6785	0.0756	0.7028	*
17	69.4	8	2.378	1.7715	*	*	0.679
18	69.4	8	2.094	1.3169	*	*	0.597
19	69.4	7	2.053	1.2719	0.2167	0.3038	0.676
20	69.4	7	2.029	0.9605	0.1083	0.2828	0.629

* Not determined.

only the major or more obvious reactions with which they are concerned. Many of the students of the chemical activities of *Aspergillus niger* have taken account of only one or at most two of the products of metabolism. The stimulating effect of various substances has been repeatedly studied by determining only the weight of the mycelium. In this study an effort has been made

to estimate all the products of metabolism in the same culture. This gives a much more adequate picture of what has occurred and affords data capable of specific interpretation.

Tables I and II are self-explanatory. Table I shows the composition of the various media and Table II the products of metabolism on the medium bearing the same number.

Some of the more important conclusions to be drawn from an analysis of these data are:

1. The proportion in which the products of the metabolism of *Aspergillus niger* appear can be varied at will.

2. Cultures which cannot be distinguished on morphological grounds give quite different results under the same conditions.

3. By a judicious selection of cultures and conditions citric acid can be varied from none at all to over 50 per cent of the sugar consumed.

4. The conditions especially favorable to the citric acid fermentation are low nitrogen supply, high concentration of sugar, and nitrogen supplied as ammonium salts rather than as nitrates.

5. When nitrogen is supplied as ammonium salts or as asparagine, iron does not stimulate the metabolic processes in any way.

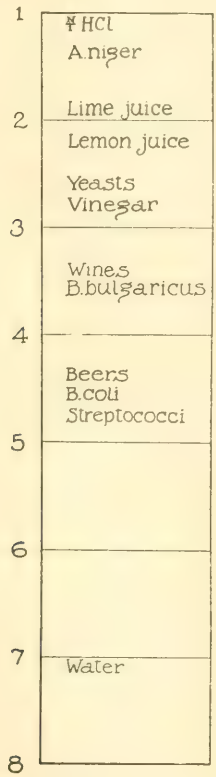
6. When nitrogen is supplied as nitrates, iron has a marked stimulating effect, especially noticeable in the increased production of carbon dioxide and weight of mycelium.

Reaction of the Medium.

Wehmer[2] and also others who have studied the citric acid fermentation worked on the supposition that the acid should be neutralized as formed or the rise in acidity would interfere with the growth of the mold. The introduction of calcium carbonate, the only practicable neutralizing substance, causes many difficulties and, it is believed the facts brought out here will show, is wholly unnecessary.

It is now clearly recognized by most workers who are concerned with the reaction of biological fluids that their hydrogen ion concentration is a much more important factor than their titratable acidity. It has long been a custom among mycologists to add some organic acid to media intended for the cultivation of

fungi. It was recognized that these acids would restrain the growth of many types of bacteria without interfering with the growth of molds. The acids most commonly used were citric and tartaric. There seems to be a general impression that the mineral acids and also oxalic acid were too toxic to be used for



TEXT-FIG. 1. pH of biological fluids.

this purpose. The basis of this impression lies in the relatively low dissociation constants of the citric and tartaric acids in comparison with the mineral acids and oxalic acid. In fact if these acids be used at the same hydrogen ion concentration, results generally show that the mineral acids are less toxic than the organic acids.

Clark and Lubs[15] give the hydrogen ion concentration of a culture of *Aspergillus niger* they examined as 2×10^{-2} N (pH 1.7). They did not attempt to determine a limit. This result together with the fact that the cultures readily produce a solution containing 10 per cent of citric acid shows that the limiting hydrogen ion concentration is very high in comparison with most organisms. It is of the same order of magnitude as that of lime and lemon juice (Text-Fig. 1).

Ten cultures of *Aspergillus niger* and one culture resembling Wehmer's *Citromyces pfefferianus* were inoculated into media made up to definite hydrogen ion concentrations with three acids, citric, oxalic, and hydrochloric. The hydrogen ion concentration was determined by the colorimetric scheme of Clark and Lubs.[16] The media employed had the following composition:

	gm.
Water	1,000 cc.
Saccharose	50
$NaNO_3$	2
KH_2PO_4	1
$MgSO_4.7H_2O$	0.2
Acid to designated pH.	

Saccharose was omitted in the medium to which citric acid was added because this acid provides a good source of carbon. Observations were made at 3 days on the oxalic and hydrochloric acid media and at 5 days on the citric acid medium. Cultures upon the medium containing citric acid grew slower than the others because citric acid is less readily consumed than saccharose. Results are shown in Table III.

This table shows that the critical pH for most of these cultures lies between 1.6 and 1.4. This is in agreement with results obtained by determining the hydrogen ion concentration of liquids fermented by Culture 28.7. The highest result determined by the hydrogen electrode was 1.46.[17]

It is interesting to note that three or four of the cultures showed a decidedly greater resistance than the others, and also that the culture showing the greatest resistance was the culture that

[15] Clark, W. M., and Lubs, H. A., *J. Bact.*, 1917, ii, 6.

[16] Clark and Lubs, *J. Bact.*, 1917, ii, 33.

[17] This determination was made by Dr. W. M. Clark of this laboratory.

had previously been selected as the best one for the citric acid fermentation.

A glance at the columns representing the pH and the per cent of acid present shows clearly the importance of considering the hydrogen ion concentration. The titratable acidity of the medium containing the citric acid at pH 1.4 is 62 times that of the medium containing the hydrochloric acid and having the same pH and the same inhibiting effect. In this connection the writer cannot resist the temptation to point out the possible bearing of these facts on the problem of preserving the juice of the citrus fruits.[18]

TABLE III.

Inhibiting Effect of Hydrogen Ion Concentration on Aspergillus niger.

	Hydrochloric acid.					Oxalic acid.					Citric acid.			
Per cent of acid...	0.087	0.098	0.113	0.138	0.168	0.25	0.37	0.50	0.62	0.75	7.5	10.0	15.0	20
pH...............	1.8	1.7	1.6	1.5	1.4	1.8	1.7	1.6	1.5	1.4	1.7	1.6	1.5	1
A. niger 28.7...	++	++	++	+	+	++	++	+	+	+	++	++	+	[illegible]
" 96 ...	++	++	++	+	+	++	++	+	+	?	++	++	+	[illegible]
" 74 ...	++	++	+	+	+	++	++	+	+	+	++	+	+	[illegible]
" 49 ...	++	++	++	+	+	++	++	+	+	+	+	+	?	[illegible]
" 50 ...	++	+	+	?	0	++	+	+	0	0	+	+	?	[illegible]
" 57 ...	++	+	?	?	0	+	+	+	?	0	+	+	+	[illegible]
" 69.4...	++	++	+	?	0	+	+	?	?	?	+	+	?	[illegible]
" 47 ...	++	+	?	0	0	+	+	?	0	0	+	+	0	[illegible]
" 111 ...	++	+	+	0	0	+	+	0	0	0	+	+	+	[illegible]
" 142 ...	++	+	?	?	0	+	+	?	?	?	+	?	0	[illegible]
Penicillium 45 ...	++	++	++	+	+	++	++	++	+	+	++	++	+	[illegible]

+ indicates positive growth; ? indicates germination; 0 indicates no development.

It is hardly practicable to consider producing a liquid containing more than 10 per cent of citric acid for the concentration of sugar required would exceed the point where the fermentation proceeds most rapidly. This concentration of citric acid does not interfere with the growth of the mold. After a concentration of 20 per cent of citric acid is reached the increase in hydrogen ion concentration is very slight in comparison to the added acid. Culture 28.7 will make considerable growth on a medium containing 40 per cent of citric acid.

[18] Will, R. T., *J. Ind. and Eng. Chem.*, 1916, viii, 78.

Wehmer[2] states that one of the chief reasons for the failure of his process was the difficulty encountered in preventing his media from becoming contaminated with organisms which interfered with the citric acid fermentation. A glance at the tables given by Clark and Lubs[19] will show that few bacteria will grow below a pH of about 3.5. It would require at least 10 per cent of citric acid to lower the pH from this point to the region where the hydrogen ion concentration would begin to interfere with growth. It is therefore clear that the fermentation can be started off at a hydrogen ion concentration that will greatly reduce the chances of infection. This is the best argument against combining the acid in the form of calcium citrate as the fermentation proceeds. Wehmer[2] states that the yield of citric acid was increased by adding calcium carbonate for some of the citric acid was thereby removed from the field of action and could not be consumed by the growing mold. This observation is not in accord with the experience of the writer. Higher yields have been obtained and in a shorter time in the absence of calcium carbonate. When the fermentation proceeds properly there is little consumption of citric acid as long as sugar is present. Even if calcium carbonate were present the substrate in immediate contact with the mold mycelium would contain free citric acid or calcium citrate in solution and the chance for the consumption of the products of fermentation would not be materially lessened.

The element of time should also be taken into consideration. Hallerbach[20] states that Wehmer's process failed because it required too long a time on a technical scale. The fermentation proceeds much more rapidly in an acid medium than in one to which calcium carbonate has been added. This is probably partly due to the reaction and partly to the disturbance of the equilibrium of salts in solution in the medium. The magnesium and phosphate radicles, both of which are essential to the growth of the mold, would be at least partially precipitated.

There is also a possibility of recovering the citric acid directly from the fermented liquor, without going through the expensive process of separating and decomposing the calcium citrate. This

[19] Clark and Lubs, *J. Bact.*, 1917, ii, 219.

[20] Hallerbach, W., Die Citronensäure and ihre Derivate, Berlin, 1911, 25.

cannot be done with lime juice because of the high percentage of sugar, pectins, and proteins.

It is possible to ferment a second and perhaps even a third or fourth batch of medium with the same mycelial felt with a considerable saving of time. If only a liquid is to be removed this can very readily be drained off and a new batch run into the fermentation pans. The removal of the products of fermentation would be a much more difficult problem if it were necessary to remove solids such as calcium carbonate or citrate.

Selection of a Medium for the Citric Acid Fermentation.

In the work recorded in the pages that immediately follow, an effort was made to determine not only the simplest salt mixture favorable for the acid fermentation of *Aspergillus niger* but also the minimum quantity of each salt necessary. In the beginning a medium was employed after the formula of Czapek's solution, which has the following composition:

	gm.
Water	1,000 cc.
Saccharose	50
$NaNO_3$	2
KH_2PO_4	1
KCl	0.5
$MgSO_4.7H_2O$	0.5
$FeSO_4.7H_2O$	0.01

In order to study the effect of nitrogen on the acid fermentation of *Aspergillus niger*, Culture 142 was grown upon a medium of the following composition:

	gm.
Water	1,000 cc.
Saccharose	50
KH_2PO_4	1
$MgSO_4.7H_2O$	0.25
KCl	0.25
$FeSO_4.7H_2O$	0.01
$NaNO_3$	Varying quantity.

The cultures were examined on the 7th day. The results in Table IV are in accord with all the other data on this particular question, and show clearly that the restriction of the supply of

nitrogen tends to increase the amount of citric acid at the expense of the end-products of metabolism.

TABLE IV.

Effect of Varying Quantities of Nitrogen on the Acid Fermentation of Aspergillus niger 142. Results Expressed in N/10 Cc. per 50 Cc. of Medium.

$NaNO_3$ per 1,000 cc.	Acidity by titration.	Oxalic acid.	Citric acid.	Weight of mycelium.
				gm.
1.2	166.9	108.1	58.3	0.398
1.4	169.3	130.6	39.7	0.500
1.6	162.2	137.5	27.2	0.548
1.8	161.0	141.0	24.4	0.634
2.0	152.4	143.5	14.5	0.606
2.2	158.2	150.9	14.1	0.616
2.4	158.0	152.5	5.7	0.643
2.6	152.9	154.9	6.5	0.637
2.8	149.2	153.2	7.8	0.644
3.0	114.8	124.1	0.0	0.695

In order to determine the quantity of potassium the medium should contain, four cultures were grown upon 50 cc. of medium of the following composition:

	gm.
Water	1,000 cc.
Saccharose	50
$NH_4H_2PO_4$	2.0
$MgSO_4$	0.2
KCl	Varying quantity.

The cultures were examined when 8 days old. For tabulation of results see Table V. The results indicate that the potassium requirements are very low, in most cases, 0.2 gm. per liter being sufficient.

In order to determine the effect of varying quantities of magnesium sulfate on the acid fermentation of *Aspergillus niger*, Culture 142 was grown upon a medium of the following composition:

	gm.
Water	1,000 cc.
Saccharose	50
$NaNO_3$	2
KH_2PO_4	1
KCl	0.25
$FeSO_4.7H_2O$	0.01
$MgSO_4.7H_2O$	Varying quantity.

TABLE V.

Effect of Varying Quantities of Potassium on the Acid Fermentation of Aspergillus niger. Results Expressed in N/10 Cc. per 50 Cc. of Medium.

Culture.	KCl per 1,000 cc.	Acidity by titration.	Oxalic acid.	Citric acid.	Weight of mycelium.
	gm.				gm.
142	0.2	115.2	52.8	45.6	0.335
	0.4	136.4	64.6	55.7	0.457
	0.6	127.4	55.2	52.0	0.382
69.4	0.2	110.4	0.0	94.3	0.535
	0.4	118.8	0.0	101.5	0.520
	0.6	97.6	14.4	63.0	0.494
28.7	0.2	143.2	10.0	117.1	0.631
	0.4	120.4	6.0	98.2	0.543
	0.6	119.2	6.8	93.8	0.522
74	0.2	159.4	17.6	133.8	0.498
	0.4	124.8	22.8	81.6	0.572
	0.6	151.2	23.8	109.2	0.492

The cultures were examined when 5 days old. From the results it appears that the most favorable quantity of $MgSO_4.7H_2O$ for a medium of the above composition lies between 0.1 and 0.2 gm. per liter (Table VI).

TABLE VI.

Effect of Varying Quantities of $MgSO_4.7H_2O$ on the Acid Fermentation of Aspergillus niger 142. Results Expressed in N/10 Cc. per 50 Cc. of Medium.

$MgSO_4$ per 1,000 cc.	Acidity by titration.	Oxalic acid.	Citric acid.	Weight of mycelium.
gm.				gm.
0.01	31.3	31.0	Trace.	0.123
0.05	72.0	75.0	"	0.394
0.10	103.5	109.8	2.1	0.521
0.15	112.8	103.8	10.8	0.551
0.20	93.3	83.3	9.4	0.547
0.25	94.3	86.8	16.5	0.460
0.30	89.0	78.0	8.8	0.456
0.40	82.5	69.5	8.6	0.436

In order to determine the effect of iron on the acid fermentation of *Aspergillus niger*, Culture 142 was grown upon a medium of the following composition:

	gm.
Water	1,000 cc.
Saccharose	50
KH_2PO_4	1
$NaNO_3$	3
$MgSO_4.7H_2O$	0.25
KCl	0.25
$FeSO_4.7H_2O$	Varying quantity.

The cultures were examined on the 7th day. No determinations were made other than total acidity. The results are shown below (50 cc. of medium were employed).

$FeSO_4.7H_2O$ per 1,000 cc., *gm.*	0.0	0.004	0.01	0.02	0.05	0.1
N/10 acidity, *cc.*	89.5	133.8	135.0	98.0	98.5	110.5

Other studies have been made on the effect of iron. In general the results were in agreement with those shown above. The addition of about 0.01 gm. of ferrous sulfate per liter to media containing nitrates stimulates the growth of mycelium and increases the rate of metabolism, especially in the earlier days of the fermentation. If the fermentation be continued for periods longer than 6 or 7 days, the unstimulated cultures tend to overtake and even to surpass the stimulated ones in total acidity.

In order to determine whether the mixture of inorganic salts, contained in Czapek's media, were not more complex than really necessary five cultures of *Aspergillus niger* were grown upon media of the composition shown below:

	gm.
Water	1,000 cc.
Saccharose	50
$NaNO_3$	2
KH_2PO_4	1
$MgSO_4.7H_2O$	0.5

No. 1. The above salts.
" 2. " " " + 0.5 KCl.
" 3. " " " + 0.5 " + 0.01 $FeSO_4.7H_2O$.
" 4. " " " + 0.5 NaCl.

The results in Table VII show that the duplication of the potassium radicle and the introduction of chlorides and iron are not only unnecessary but in most cases even unfavorable to the acid fermentation of *Aspergillus niger*.

TABLE VII.

Results Expressed in Cc. N/10 *per 50 Cc. of Medium.*

Culture.	No. of medium.	Acidity.	Oxalic acid.	Citric acid.	Oxalic and citric.	Weight of mycelium.
						gm.
142	1	148.8	91.0	61.8	152.8	0.506
	2	142.8	103.6	44.0	147.6	0.469
	3	152.0	118.6	40.8	157.4	0.495
	4	137.6	88.6	60.4	149.0	0.486
69.4	1	135.6	41.4	94.8	136.2	0.462
	2	111.4	38.0	83.5	121.5	0.472
	3	79.6	42.4	42.9	85.3	0.454
	4	103.0	42.0	75.5	117.5	0.461
28.7	1	140.0	67.0	66.1	133.1	0.511
	2	82.8	43.2	35.7	78.9	0.521
	3	78.6	32.6	47.9	80.5	0.570
	4	92.8	37.0	51.9	88.9	0.532
74	1	115.4	72.0	47.3	119.3	0.438
	2	39.2	27.0	10.4	37.4	0.458
	3	45.4	32.0	19.0	41.0	0.493
	4	44.4	34.8	12.4	37.2	0.438
96	1	91.2	44.4	45.9	90.1	0.597
	2	78.8	34.0	44.3	78.3	0.549
	3	71.2	34.4	42.1	76.5	0.617
	4	81.4	36.8	43.0	79.8	0.591

From the experience gained in all of the foregoing studies on the mineral nutrition, the reaction of media, and the general equation of metabolism of *Aspergillus niger*, and from many experiments not reported in this paper it was concluded that the most suitable medium for conducting the citric acid fermentation with *Aspergillus niger* should have about the following composition per 1,000 cc.

	gm.
Saccharose	125 –150
NH_4NO_3	2.0 –2.5
KH_2PO_4	0.75–1.0
$MgSO_4.7H_2O$	0.20–0.25
HCl to pH 3.4 – 3.5 (5 – 4 cc. N/5).	

The medium proposed by Wehmer[21] contained "a small quantity of mineral salts (NH_4NO_3, KH_2PO_4, and $MgSO_4$)." Zahorski[5] used "small amounts of nutrient salts such as ammonium nitrate, potassium phosphate, and magnesium sulfate."

The addition of hydrochloric acid to the mixture of inorganic salts proposed above has several advantages. It raises the hydrogen ion concentration to a point that makes complete sterilization possible at a single heating in steam at atmospheric pressure for 30 minutes. As previously pointed out, it greatly reduces the dangers of infection of the liquors with organisms which might interfere with the citric acid fermentation without inhibiting the growth of the mold with which the liquors are inoculated.

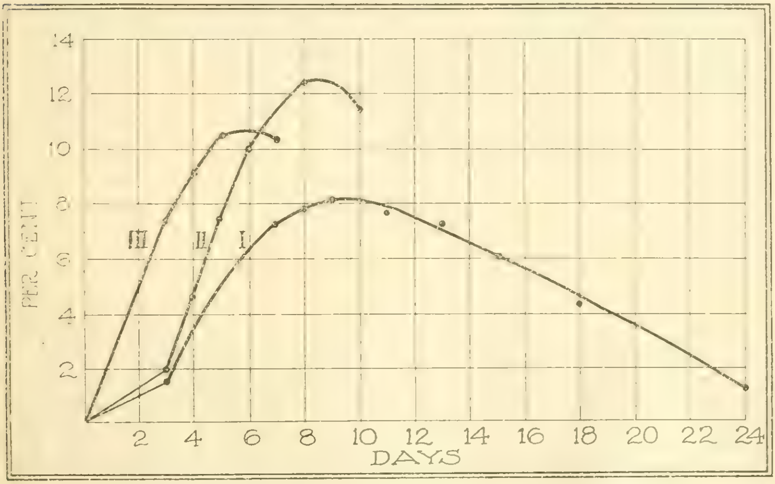
Many fermentations have been conducted in Erlenmeyer flasks on this medium. The general course of the fermentation is quite similar. There is little development of acid during the first 2 or 3 days. When a vigorous mycelial felt has developed the rise in acidity is very rapid, about 2 per cent in 24 hours, until the 7th or 8th day. After remaining nearly constant for 2 or 3 days the acidity begins to decline. When the fermentation proceeds properly the mold does not spore but remains white as shown in Figs. 3 and 4.

The course of the fermentation under varying conditions is shown by the curves in Text-fig. 2. Curve I shows the course of the development of acid in a 12.5 per cent cane sugar solution. 100 cc. of the medium were contained in a 300 cc. Erlenmeyer flask. 5 cc. were removed at intervals and the acidity was determined by titration. Curve II shows the development of acid in a 15 per cent cane sugar solution. Curve III shows the course of the fermentation in a shallow pan (Fig. 5) containing 1 liter of a 15 per cent cane sugar solution. The mycelium had been developed in a previous fermentation and the rise in acidity began at once.

[21] Wehmer, C., U. S. Patent No. 515,033, Feb. 20, 1894.

With Culture 28.7 the acidity generally reaches about 10 per cent on the 8th day. Not infrequently the acidity is as high as 12 per cent although sometimes it fails to rise above 8 per cent. Along with the citric acid there are generally traces of oxalic acid and sometimes this latter acid may account for 3 or 4 per cent of the total acidity. The removal of this oxalic acid by partial neutralization of the fermented liquors with calcium carbonate would probably present no serious difficulties.

The variability of the fermentation under what appears to be identical conditions is a difficulty that has not been entirely overcome. Wehmer[2] stated that the greatest difficulty he had to



Text-Fig. 2. The course of the citric acid fermentation.

contend with, aside from the infection of his liquors with undesirable organisms, was the varying fermentative power of his cultures (*Variabilität des Garvermögens*). Throughout this study it has been emphasized that a complex biological reaction is involved which results in a number of products and which cannot be expressed by a simple equation, representing the oxidation of a sugar to citric acid. All of the conditions that may influence the course of the fermentation are not within the control of the experimenter. Good results can be had only by the adoption of conditions that prove successful and can be duplicated with a high degree of uniformity.

While most of the experiments have been conducted in Erlenmeyer flasks of various sizes, the fermentation can be conducted with equal success in shallow pans. 1 liter of a 15 per cent saccharose solution was fermented in each of three pans (Fig. 4). The fermentation was continued for 8 days. About 800 cc. of liquor could be recovered from each pan by pressing out the mycelium in a hand filter press. Analytical data on the liquid from each pan are shown in the table below:

Pan.	Citric acid by titration.	Calcium citrate in 25 cc. by precipitation.	Calcium citrate.	Citric acid calculated from calcium citrate.	Sugar remaining in the liquor.
	per cent	*gm.*	*per cent*	*per cent*	*per cent*
1	11.54	3.8789	15.52	10.35	3.95
2	11.51	3.8209	15.28	10.19	4.69
3	11.00	3.7673	15.07	10.05	5.93

Already many substances of great technical value such as ethyl alcohol, acetic acid, butyric acid, and lactic acid are prepared by biochemical processes. In a recent paper on the chemical activities of yeasts, molds, and bacteria, Ehrlich[22] concluded with the prophecy that in time we would have a great chemical fermentation industry in which many substances would be prepared which are now manufactured by expensive synthetic methods. Many such substances are known to occur as metabolic products of microorganisms. The painstaking investigation of all the conditions favoring the production of such substances will lay the only sure foundations for the development of a chemical fermentation industry. It is the hope of the writer that the work here recorded may prove a definite contribution to this much neglected but promising field of scientific endeavor.

EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. Ten strains of *Aspergillus niger*. Showing sporification on a medium containing no iron.

FIG. 2. Ten strains of *Aspergillus niger*. Showing failure to spore on a medium containing iron.

[22] Ehrlich, F., *Z. angew. Chem.*, 1914, xxvii, 48.

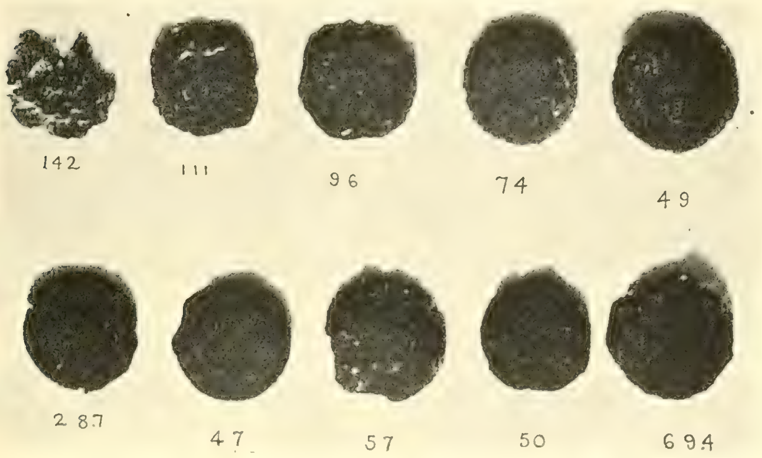


Fig. 1.

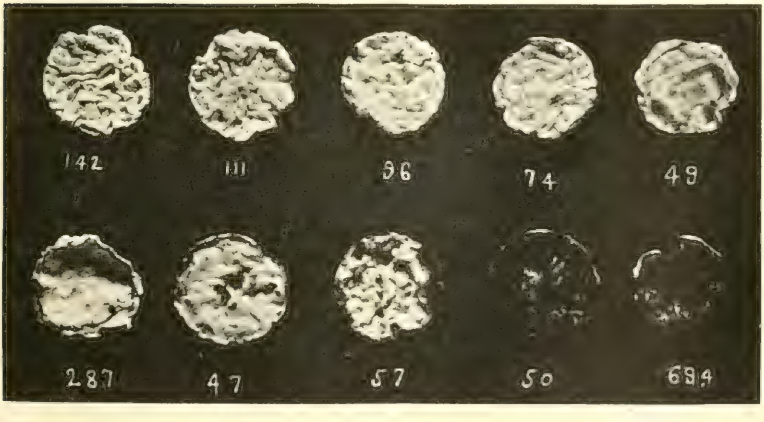


Fig. 2.

(Currie: Citric Acid Fermentation.)

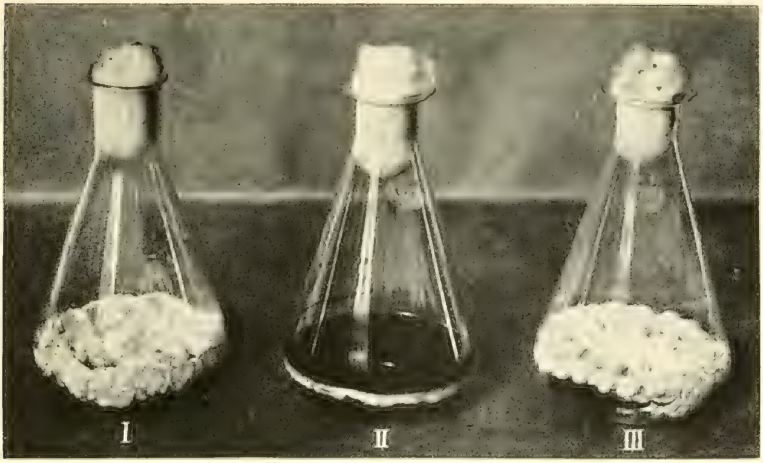


FIG. 3.

FIG. 4.

FIG. 5.

(Currie: Citric Acid Fermentation.)

Plate 2.

Fig. 3. Culture 28.7. I and III show the appearance of the culture when the citric acid fermentation proceeds properly. II shows the same culture on a medium which favors sporification.

Fig. 4. Culture 28.7 fermenting 1 liter of liquid in a shallow pan.

Fig. 5. Culture 28.7. Showing the appearance of the mycelium at the beginning of the third fermentation. Note the increased thickness and the deeply wrinkled structure of the mycelium.

URACIL-CYTOSINE DINUCLEOTIDE.

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(Received for publication, May 7, 1917.)

It has been shown that yeast nucleic acid is composed of the groups of four mononucleotides joined to one another through their carbohydrate groups.[1]

When the nucleic acid is heated with *ammonia* it produces adenine-uracil dinucleotide,[2] evidently by hydrolytic rupture of its central nucleotide linkage as is indicated in the formula below (upper rectangle).

We will now show that when the nucleic acid is heated with *mineral acid* its central nucleotide linkage is not disturbed but the two terminal nucleotide linkages are broken and uracil-cytosine dinucleotide is formed, as is indicated in the formula below (lower rectangle).

The difference in the behavior of yeast nucleic acid toward ammonia on the one hand and toward mineral acid on the other hand shows a remarkable specific difference between hydroxyl ions and hydrogen ions as hydrolytic agents; especially as the initial substance and all of the hydrolytic products are easily soluble.

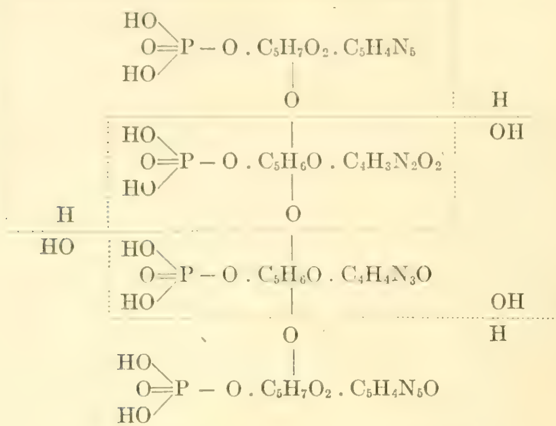
Uracil-cytosine dinucleotide produces both uracil and cytosine but neither guanine nor adenine. It likewise forms both pyrimidine nucleosides but neither of the two purine nucleosides, and yields no easily split phosphoric acid.

When an aqueous solution of the dinucleotide is treated with an alcoholic solution of brucine, a brucine salt is formed which crystallizes from hot water in macroscopic individual needles having the composition required for the formula $C_{18}H_{25}N_5P_2O_{16}$.-

[1] Jones, W., and Read, B. E., *J. Biol. Chem.*, 1917, xxix, 123.

[2] Jones and Read, *J. Biol. Chem.*, 1917, xxix, 111.

$4(C_{23}H_{26}N_2O_4).14H_2O$. As the brucine salt contains four equivalents of brucine, the mononucleotide groups that compose the dinucleotide must be united to one another through their carbohydrate groups.[2] We formerly inferred this mode of nucleotide linkage[1] from a study of the rate at which phosphoric acid is liberated from *yeast nucleic acid* by hydrolysis with mineral acid.



Formula for yeast nucleic acid showing how the two dinucleotides are formed.

EXPERIMENTAL.

Preparation and Properties of Uracil-Cytosine Dinucleotide.

Commercial yeast nucleic acid in portions of 50 gm. was boiled with 250 cc. of 5 per cent sulfuric acid for $1\frac{1}{2}$ hours under an inverted condenser. These are the conditions which our previously reported experiments show to be most favorable for the complete liberation of the purine-phosphoric acid with a minimum destruction of pyrimidine nucleotides.[3] The product was treated with freshly precipitated silver oxide in such amount that a drop of the fluid formed a brown precipitate with sodium hydroxide, and after standing until perfectly cold the purine-silver compounds were filtered off. The solution was treated with warm saturated barium hydroxide until faintly alkaline to litmus and the pre-

[3] Jones, *J. Biol. Chem.*, 1916, xxiv, p. iii.

cipitated silver compound of the dinucleotide was suspended in hot water and decomposed with sulfuretted hydrogen. After treatment of the filtrate from silver sulfide with sulfuric acid for the removal of a trace of barium, the fluid was evaporated to a syrup at 50° under diminished pressure and the dinucleotide was precipitated with absolute alcohol. The precipitate easily hardens with absolute alcohol to a granular white powder.

The dinucleotide is easily soluble in cold water and dextrorotatory to polarized light.

1.5 gm. (7 per cent moisture) dissolved in 15 cc. of water gave a reading of + 2.82° in a 2 dm. tube. $[\alpha]_D = +15.0°$.

When boiled with twenty parts of 5 per cent sulfuric acid, the dinucleotide does not produce a trace of either guanine or adenine, and loses its phosphoric acid very slowly.

1.0362 gm. dried at 105° was boiled for 3 hours with 20 cc. of 5 per cent sulfuric acid. The cooled product was made alkaline with ammonia but no guanine was deposited even after standing for several hours in ice water. The warmed solution was then treated with magnesia mixture and after standing over night the precipitated magnesium ammonium phosphate was filtered off and weighed.

	mg.
Dinucleotide used	1,036.2
$MgNH_4PO_4 \cdot 6H_2O$ obtained	64.7
Calculated amount per gm.-hr.	20.8

In our reported studies of the rate at which phosphoric acid is liberated from yeast nucleic acid[3] we ascribed 20 mg. of magnesium ammonium phosphate per gm.-hour to the pyrimidine nucleotides.

The filtrate from magnesium ammonium phosphate gave no purine precipitate with ammoniacal silver nitrate.

The crude dinucleotide dried at 105° gave the following analytical results. The calculated values are for the formula $C_{18}H_{25}N_5P_2O_{16}$.

I. 0.2147 gm. gave 0.2805 gm. CO_2 and 0.0812 gm. H_2O.
II. 0.2804 " required 8.47 cc. H_2SO_4 (1 cc. = 0.0037 N).
III. 0.3267 " " 9.81 " " (1 " 0.0037 "
IV. 0.3587 " gave 0.1081 gm. $Mg_2P_2O_7$.
V. 0.4157 " " 0.1264 " "

	C	H	N	P
Calculated	34.34	3.97	11.13	9.86
Found.				
I	35.63	4.20		
II			11.17	
III			11.11	
IV				8.42
V				8.49

Preparation of the Uridine from Uracil-Cytosine Dinucleotide.

25 gm. of dinucleotide were heated in an autoclave for 2 hours at 140° with 140 cc. of 2 per cent ammonia. No guanosine was deposited even after the cooled product had stood several hours in ice water, and picric acid produced no precipitate of adenosine picrate. The fluid was transferred to a vacuum distilling apparatus, evaporated at 50° under diminished pressure to about 30 cc., and after the addition of 300 cc. of absolute alcohol, the solution was saturated with dry hydrochloric acid gas. Uridine was then isolated by the method of Levene and La Forge.[4] After crystallization from hot alcohol the substance was obtained in large snow-white crystals which melted at 158–159° (corrected).

I. 0.3451 gm. required 10.61 cc. H_2SO_4 (1 cc. = 0.0037 gm. N).
II. 0.3366 " " 10.41 " " (1 " 0.0037 " "

	N
Calculated for $C_9H_{12}N_2O_6$	11.48
Found.	
I	11.38
II	11.44

From 25 gm. of dinucleotide 5.2 gm. of pure uridine were obtained.

The mother liquor from uridine after evaporation of the alcohol gave qualitative tests for cytidine; *i.e.*, produced a crystalline nitrate with nitric acid and a picrate with picric acid crystallizable from alcohol.

[4] Levene, P. A., and La Forge, F. B., *Ber. chem. Ges.*, 1912, xlv, 613.

Preparation of Uracil and Cytosine from Uracil-Cytosine Dinucleotide.

20 gm. of dinucleotide were heated with 100 cc. of 25 per cent sulfuric acid in an autoclave for 3 hours at 140°. After cooling, the black pigment was filtered off and the diluted red solution was treated with barium hydrate for the removal of sulfuric and phosphoric acids. The fluid was evaporated at 50° under diminished pressure and treated with hot saturated picric acid solution as long as a drop of the fluid formed a precipitate with cold picric acid. A copious precipitate of cytosine picrate was formed (probably 6 or 8 gm.). A portion of the picrate upon recrystallization from hot water melted at 265–270° (uncorrected). Another portion of cytosine picrate was dissolved in hot water, acidified to Congo red with sulfuric acid, and after cooling somewhat was shaken out with ether for the complete removal of picric acid. The solution then was treated for cytosine by the silver-barium method. The final solution of cytosine sulfate was acidified to Congo red with sulfuric acid and evaporated to a small volume on the water bath. After cooling, it was strongly acidified with sulfuric acid and treated with two volumes of absolute alcohol. Cytosine sulfate was almost immediately thrown down in glistening crystal grains which appeared under the microscope as colorless transparent tables.

This is the best method of preparing cytosine sulfate $(C_4H_5N_3O)_2$-$H_2SO_4.2H_2O$. A possible trace of uracil is eliminated, the coloring matter remains in the alcoholic mother liquor, and the formation of basic cytosine sulfate is avoided.

I. 0.2591 gm. lost 0.0264 gm. at 120°, gave 0.1686 gm. $BaSO_4$, and required 16.54 cc. of H_2SO_4 (1 cc. = 0.0037 gm. N).

II. 0.2193 gm. required 13.92 cc. H_2SO_4.

	H_2O	H_2SO_4	N
Calculated for $(C_4H_5N_3O)_2H_2SO_4.2H_2O$	10.11	27.24	23.60
Found.			
I	10.19	27.37	23.62
II			23.49

From the pure sulfate, the pure picrate was prepared in transparent yellow needles which decomposed and melted sharply at 260–261° (uncorrected).

The original filtrate from the crude cytosine picrate was treated with sulfuric acid and ether for the removal of picric acid, and uracil was isolated by the silver-barium method in characteristic needle clusters.

0.2651 gm. required 17.87 cc. of H_2SO_4 (1 cc. = 0.0037 gm. N).

	N
Calculated for $C_4H_4N_2O_2$	25.00
Found	24.94

The Brucine Salt of Uracil-Cytosine Dinucleotide.

1 part of dinucleotide in 5 parts of water was treated with 2.4 parts of brucine in 5 parts of hot alcohol. The crystalline brucine salt was precipitated almost immediately. After standing over night the crystals were filtered off, washed with cold water, and then with hot alcohol. The product thus obtained was recrystallized from 100 parts of hot water. From 4.5 gm. of dinucleotide were obtained 8.4 gm. of brucine salt which on recrystallization gave 5.1 gm. of beautifully crystalline needles. These crystals do not change in chemical composition when recrystallized from hot water and washed with hot alcohol.

When heated in a capillary tube the salt contracts and recedes from the sides of the tube at 170–174° and melts at 175°. The same conduct is exhibited by the brucine salt of adenine-uracil dinucleotide and is evidently a brucine phenomenon as it occurs at the melting point of brucine (175°).

Analysis of the Brucine Salt.

The methods used are described in our former article[2] except that in the estimation of brucine no correction was added for the part extracted by chloroform.

The carbon determinations were difficult because carbon particles became included in the residual phosphoric acid and could be burned out only by very long heating in an oxygen current.

The calculated percentages are for the formula $C_{18}H_{25}N_5P_2O_{16}$.-4($C_{23}H_{26}N_2O_4$).14H_2O.

I. 1.1277 gm. lost 0.1160 gm. at 125°.
II. 0.5486 " " 0.0552 " " 125°.
III. 0.2992 " gave 0.5858 " CO_2 and 0.1771 gm. H_2O.
IV. 0.3006 " " 0.5861 " " " 0.1666 " "
V. 0.4050 " " 27.4 cc. N at 27° and 758 mm.
VI. 0.4268 " " 28.7 " " " 25.5° " 759 "
VII. 0.9262 " " 0.0833 gm. $Mg_2P_2O_7$.
VIII. 1.1013 " " 0.1004 " "
IX. 0.3912 " " 0.2450 " brucine.

	H_2O	C	H	N	P	Brucine.
Calculated	10.26	53.72	6.39	7.41	2.52	64.14
Found.						
I	10.29					
II	10.06					
III		53.39	6.58			
IV		53.20	6.16			
V				7.45		
VI				7.47		
VII					2.51	
VIII					2.54	
IX						62.63

Six years ago Levene and Jacobs[5] obtained from yeast nucleic acid, after acid hydrolysis, a barium preparation and a sodium preparation which they stated to be mixtures of salts of the two pyrimidine mononucleotides. However, the substance which we have described as a dinucleotide is certainly not a mixture of the two mononucleotides. The isolation of the two mononucleotides in equivalent quantities which form an isomorphous mixture of the two brucine salts in exactly equivalent quantities would be so unusual that it scarcely deserves consideration. But aside from this, we are in possession of conclusive evidence to show that at least one of the pyrimidine mononucleotides (uracil mononucleotide) would have been entirely eliminated in the process which we employed for the preparation of uracil-cytosine dinucleotide.

[5] Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, 1911, xliv, 1027.

GUANINE MONONUCLEOTIDE (GUANYLIC ACID) AND ITS PREPARATION FROM YEAST NUCLEIC ACID.

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(Received for publication, May 11, 1917.)

It has been shown that guanine mononucleotide can be produced from yeast nucleic acid by the action of ferments[1] and the probable origin of the guanylic acid of animal glands has been thus explained.[2]

It is the purpose of this paper to describe the preparation of guanine mononucleotide by a chemical procedure which consists simply in heating the nucleic acid with ammonia. Hydrolysis occurs as represented in the diagram below, and the product is free from glandular constituents so that the mononucleotide can be easily isolated in comparatively great quantity and in perfect chemical purity.

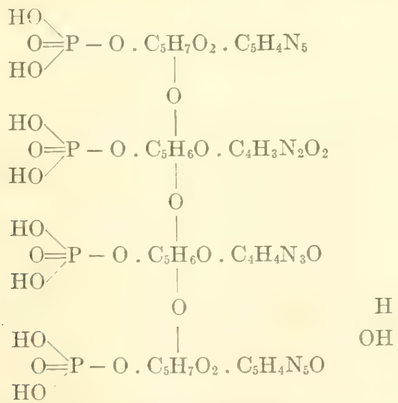

Diagram showing the formation of guanine mononucleotide from yeast nucleic acid.

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[1] Jones, W., and Richards, A. E., *J. Biol. Chem.*, 1914, xvii, 71.

[2] Jones and Richards, *J. Biol. Chem.*, 1915, xx, 25.

Up to the present time free guanine mononucleotide has not been prepared in pure condition, the substance being best known in the form of its derivatives. As the properties of the pure substance, one of which serves in its purification, are quite characteristic they are described in some detail, and the identity of guanine mononucleotide with the guanylic acid of glands is thoroughly established.

EXPERIMENTAL.

Decomposition of Yeast Nucleic Acid with Ammonia and Separation of the Products.

Portions of 100 gm. of yeast nucleic acid were heated with 530 cc. of 2.5 per cent ammonia in an autoclave at 115° for 1½ hours. After the product had cooled and while still alkaline with ammonia it was treated with 530 cc. of absolute alcohol and allowed to stand until the supernatant fluid had become perfectly clear. The solution, which contains adenine-uracil dinucleotide (already described),[3] was filtered and the gray precipitate, consisting principally of the ammonium salt of guanine mononucleotide, was dissolved in hot water, filtered from a trace of insoluble flocculent material with a hot water funnel, and after the addition of a few drops of ammonia the cooled fluid was treated with two volumes of absolute alcohol. By alternate solution in hot water and precipitation with alcohol the ammonium salt of guanine mononucleotide was thus freed from every trace of material that contains an adenine group. The effectiveness of the separation, however, must be ascertained by hydrolysis of a small portion of the substance with sulfuric acid and then testing for adenine. The ammonium salt is finally filtered with a pump and dried by grinding with absolute alcohol.

Preparation of the Pure Guanine Mononucleotide from the Crude Ammonium Salt.

A solution of the ammonium salt in hot water was acidified with acetic acid and treated hot with neutral lead acetate as long as a precipitate was formed. After cooling, the lead salt was filtered off with a pump, suspended in hot water, and decomposed with sulfuretted hydrogen. The filtrate from lead sulfide was

[3] Jones, W., and Read, B. E., *J. Biol. Chem.*, 1917, xxix, 111.

evaporated to a small volume at 45° under diminished pressure; upon cooling the concentrated solution in ice water, guanine mononucleotide was deposited. Upon repeated solution of the substance in hot water and deposition by cooling, every trace of soluble material can be removed, but the product still contains a small quantity of some insoluble substance which can be separated as follows. A concentrated solution of the mononucleotide in hot water is made alkaline with ammonia, boiled, filtered, acidified with acetic acid, and precipitated with lead acetate. The lead compound is decomposed with sulfuretted hydrogen, and the filtrate from lead sulfide is evaporated at 45° as described above. Without filtering, the cooled concentrated material is treated with absolute alcohol and the precipitated guanine mononucleotide is washed and dried with absolute alcohol. The substance thus obtained is a snow-white amorphous powder, difficultly soluble in cold water, but easily soluble in warm water, forming a solution from which part of the guanine mononucleotide is deposited on cooling in ice water. The substance is levorotatory to polarized light.

2.4 gm. in 50 cc. of water gave a reading of −0.23°, in a 2 dm. tube.
$[\alpha]_D = -2.4°$

A preparation dried to a constant weight at 110° gave the following analytical data. The calculated values are for the formula $C_{10}H_{14}N_5O_8P$.

I. 0.3939 gm. gave 0.4757 gm. CO_2 and 0.1386 gm. H_2O.
II. 0.2326 " required 12.11 cc. H_2SO_4 (1 cc. = 0.0037 gm. N).
III. 0.1957 " " 10.12 " " (1 " = 0.0037 " ").
IV. 0.4378 " gave 0.1341 gm. $Mg_2P_2O_7$ (total).
V. 0.8507 " " 0.5693 " $MgNH_4PO_4.6H_2O$ (total).
VI. 0.3448 " " 0.0981 " $Mg_2P_2O_7$ (partial).
VII. 0.3487 " " 0.1396 " guanine.

	C	H	N	P total.	P partial.*	Guanine.
Calculated.....	33.06	3.86	19.28	8.54	8.54	41.60
Found.						
I........	32.93	3.91				
II........			19.26			
III........			19.13			
IV........				8.55		
V........				8.47		
VI........					7.95	
VII........						40.04

* Jones, W., *J. Biol. Chem.*, 1916, xxiv, p. iii.

Preparation of Guanosine from Guanine Mononucleotide.

3.9 gm. of guanine mononucleotide were heated in an autoclave with five volumes of 2.5 per cent ammonia at 140° for 2 hours. After the product had stood over night in the ice chest, the guanosine was filtered off and recrystallized from hot water with the use of animal charcoal. 2.17 gm. of pure guanosine were obtained in long transparent needles.

I. 0.4647 gm. of crystalline substance lost 0.0531 gm. at 110°.
II. 0.1993 " dried at 110° required 13.43 cc. H_2SO_4 (1 cc. = 0.0037 gm. N).

	H_2O	N
Calculated	11.29	24.74
Found.		
I	11.43	
II		24.93

The Brucine Salt of Guanine Mononucleotide.

Portions of 1 gm. of guanine mononucleotide dissolved in 3 cc. of hot water were treated with 2.4 gm. of brucine dissolved in 5 cc. of hot absolute alcohol. The crystalline brucine salt was formed immediately; after standing it was filtered off, thoroughly washed with cold water, and then with hot alcohol.

From 1 gm. of nucleotide, 2.8 gm. of brucine salt were obtained. This was recrystallized from hot water and washed with hot alcohol. It melted at 203° (uncorrected), and when dried by exposure to the air contained 7 molecules of water of crystallization. (The brucine salts of the dinucleotides contain 14 molecules of water of crystallization.) On standing in a vaccum desiccator with sulfuric acid it rapidly loses weight which is slowly taken up again upon exposure to the air. This behavior is very characteristic of the brucine salt.

Analysis of the substance gave the following data. All determinations were made with material heated to a constant weight at 110° except the determination of moisture.

I. 6.8847 gm. lost 0.6755 gm. heated at 110°.
II. 0.9311 " after oxidation of organic matter gave 0.1947 gm. $MgNH_4PO_4.6H_2O$.
III. 1.1350 " after oxidation of organic matter gave 0.2462 gm. $MgNH_4PO_4.6H_2O$.
IV. 0.3689 " gave 35.3 cc. nitrogen gas, at 23° and 766 mm.
V. 0.3475 " " 33.6 " " " " 23° " 765.5 "

	H_2O	P total.	N
Calculated	9.89	2.69	10.94
Found.			
I	9.81		
II		2.64	
III		2.74	
IV			10.87
V			10.97

The calculated moisture values are for the formula $C_{10}H_{14}N_5O_8P.2(C_{23}H_{26}N_2O_4).7H_2O$.

When heated for an hour with twenty parts of 10 per cent sulfuric acid the substance liberated its entire phosphoric acid, as is to be expected from a purine nucleotide. The found values are somewhat below those required. This is because comparatively large volumes of fluid were necessary in dealing with the brucine salt, and the error is probably due to the loss of a few mg. of magnesium ammonium phosphate in the mother liquor.

I. 1.0875 gm. dried at 110° gave 0.2142 gm. $MgNH_4PO_4.6H_2O$.
II. 0.7244 " " " " " 0.1373 " "

	P partial.
Calculated	2.69
Found.	
I	2.49
II	2.39

When heated with 10 per cent sulfuric acid the brucine salt produces guanine very rapidly, the liberation being complete within an hour. The determinations were made by treating the product with ammonia, filtering off the mixture of brucine and guanine, and washing out the brucine with hot alcohol. The found values are naturally a little lower than those required.

I. 0.7249 gm. gave 0.0887 gm. guanine.
II. 0.8444 " " 0.0998 " "

	Guanine.
Calculated	13.12
Found.	
I	12.23
II	11.82

A specimen of free guanine mononucleotide was prepared from the pure recrystallized brucine salt. The pure substance ob-

tained in this way, however, differs neither in its properties nor in its chemical composition from the guanine mononucleotide described.

Guanylic Acid.

In order to establish thoroughly the identity of guanine mononucleotide with the guanylic acid[4] obtainable from animal glands, a number of preparations of the latter substance were made for comparison. The chemical properties and solubilities of free guanylic acid as well as those of its brucine salt were found to agree in every respect with the properties stated above for guanine mononucleotide.

Guanylic Acid of Ox Pancreas.—β-Nucleoprotein of the pancreas was first prepared by Hammarsten's method;[5] from this the potassium salt of guanylic acid was obtained by the process which Ivar Bang[4] describes. This substance was dissolved in water, treated with lead acetate, and from the precipitated lead salt free guanylic acid was prepared by the process described above for guanine mononucleotide.

The free acid is soluble in warm water and is deposited when its warm solution is cooled in ice water. Its entire phosphoric acid is easily split. The brucine salt melts at 203° (uncorrected), partially loses its water of crystallization in a vacuum desiccator over sulfuric acid, and regains the loss on subsequent exposure to the air. It is soluble in about 100 parts of cold water.

The following analyses of the brucine salt were made with material dried to a constant weight at 118°C.

I. 0.3107 gm. gave 30.4 cc. nitrogen at 26.5° and 765 mm.
II. 0.9612 " after complete oxidation gave 0.2015 gm. $MgNH_4PO_4 \cdot 6H_2O$.
III. 1.1493 " after hydrolysis for $\frac{3}{4}$ hour with 10 per cent H_2SO_4 gave 0.2208 gm. $MgNH_4PO_4 \cdot 6H_2O$, and 0.1464 gm. guanine.

	N	P total.	P partial.	Guanine.
Calculated	10.94	2.69	2.69	13.12
Found.				
I	10.90			
II		2.64		
III			2.42	12.74

[4] Bang, I., *Z. physiol. Chem.*, 1910, lxix, 167.
[5] Hammarsten, O., *Z. physiol. Chem.*, 1894, xix, 19.

A specimen of guanylic acid of ox pancreas was prepared by the mercuric sulfate method as described by Levene and Jacobs.[6] From this as a starting point free guanylic acid was prepared by means of its lead salt. The substance was found identical in all respects with the guanylic acid of the ox pancreas described above. The brucine salt melted at 201°.

I. 0.4323 gm. dried brucine salt gave 41.2 cc. N at 21° and 761 mm.
II. 1.0938 " gave after complete oxidation 0.2293 gm. $MgNH_4PO_4 \cdot 6H_2O$.
III. 1.0043 " gave after heating 1 hour with 10 per cent H_2SO_4, 0.2023 $MgNH_4PO_4 \cdot 6H_2O$, and 0.1291 gm. guanine.

	N	P total.	P partial.	Guanine.
Calculated	10.94	2.69	2.69	13.10
Found.				
I	10.86			
II		2.64		
III			2.53	12.86

Guanylic Acid of Pig Pancreas.—This was prepared from the β-nucleoprotein of the pancreas as described above for ox pancreas. Its brucine salt melted at 203° (uncorrected).

I. 0.6549 gm. after complete oxidation gave 0.1324 gm. $MgNH_4PO_4 \cdot 6H_2O$.
II. 0.6731 " after hydrolysis with 10 per cent H_2SO_4 for 1 hour gave 0.1438 gm. $MgNH_4PO_4 \cdot 6H_2O$, and 0.0724 gm. guanine.

	P total.	P partial.	Guanine.
Calculated	2.69	2.69	13.10
Found.			
I	2.55		
II		2.68	10.79

[6] Levene, P. A., and Jacobs, W. A., *J. Biol. Chem.*, 1912, xii, 421.

THE ESTIMATION OF CHLORIDES IN BODY FLUIDS.

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(Received for publication, May 3, 1917.)

The estimation of chlorides in body fluids has been carried out in these laboratories for some time past by the method of McLean and Van Slyke,[1] a method so simple and accurate that any comment on it would, in ordinary times, be unnecessary. The authors, however, insist on the use of "Merck's blood charcoal reagent," a product which at present times cannot be bought, at any rate by us, on open market. As it is our belief that the experience of these laboratories is not an isolated one, and that other laboratories may have reached, or may be reaching the end of their stock of this particular reagent, we venture to present an alternative method for the removal of the blood proteins, which is just as rapid and efficient as that proposed by McLean and Van Slyke, and requires the use of reagents which are not of any highly specialized character, and which at the same time disturbs as little as possible the routine of the McLean and Van Slyke method. The alternative method we propose for the removal of the blood proteins is not a new one, and it is merely in its application to the estimation of chlorides that it possesses any point of novelty.

The use of copper sulfate and alkali for the removal of proteins in milk is one of the well recognized methods, in the estimation of its lactose content.[2] Its application, however, to the estimation of the glucose content of blood has led to incorrect results, due to the high alkalinity required. This objection does not apply to the estimation of chlorides. The method we have adopted is as follows.

[1] McLean, F. C., and Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxi, 361.
[2] Woodman, A. G., Food Analysis, New York, 1915, 117.

2 cc. of oxalated blood plasma are pipetted into a 20 cc. volumetric flask containing 10 cc. of distilled water; 0.5 cc. of a 7 per cent copper sulfate solution is added, together with 2 cc. of $\frac{N}{50}$ sodium hydrate. The contents of the flask are well mixed, two drops of caprylic alcohol added, and the flask is made up to the mark. The addition of the caprylic alcohol at this point enables the flask to be made up to the mark with certainty, and later on prevents the creeping and consequent loss of precipitate in the narrow stem of the volumetric flask during the heating period. The flask is then loosely stoppered and heated for a period of 10 minutes in a boiling water bath. Two or three times during the heating period (especially at the beginning) the flask is removed from the bath, the stopper tightened, and the precipitate and fluid are mixed by inverting the flask three or four times. At the end of the heating period the flask is removed from the bath, and allowed to cool to room temperature. This may be hastened by cooling in running water. The liquid is next filtered through a dry folded filter into a clean dry beaker or flask. About 15 cc. of filtrate are usually obtained, which should be perfectly clear, though colored faintly blue by the slight excess of copper. 10 cc. of the filtrate are then taken in a 25 cc. volumetric flask, and to it are added 5 cc. of 10 per cent magnesium sulfate solution and 5 cc. of the standard silver nitrate solution used by McLean and Van Slyke. The contents of the flask are made up to the mark, and on mixing, the silver chloride should readily coagulate in a few minutes. Occasionally we have had to use a drop or two of caprylic alcohol at this point to assist coagulation, as recommended by McLean and Van Slyke. From this point the procedure is exactly as that described by McLean and Van Slyke.

The above directions also hold for the estimation of chlorides in pleural, hydrocele, and cerebrospinal fluids. When using whole blood, however, it is necessary to use 1 cc. of copper sulfate and 4 cc. of $\frac{N}{50}$ sodium hydrate, the remaining directions being the same.

It will thus be seen that, save for the removal of the proteins, the method is almost identical with that of McLean and Van Slyke, and, needless to say, all those precautions in the use of instruments, etc., which have been indicated by these authors are also requisite in the alternative method.

In the following tables we have compared the results obtained by the new method with those of the original method of McLean and Van Slyke.

TABLE I.

Blood Plasma.

Diagnosis.	$\frac{M}{58.5}$ KI.		NaCl per cc. of sample.	
	New method.	McLean and Van Slyke.	New method.	McLean and Van Slyke.
	cc.	*cc.*	*mg.*	*mg.*
Brain tumor	3.40	3.40	5.75	5.75
" "	3.40	3.37	5.75	5.78
Cerebrospinal lues	3.37	3.35	5.78	5.80
" "	3.35	3.30	5.80	5.87
Tabes	3.35	3.35	5.80	5.80
Bronchitis	3.35	3.30	5.80	5.87
Chronic arthritis	3.35	3.30	5.80	5.87
Acute rheumatic fever	3.45	3.40	5.68	5.75
Splenomyelogenous leukemia	3.35	3.40	5.80	5.75
Orthostatic albuminuria	3.35	3.35	5.80	5.80
Myocarditis, failure of compensation	3.17	3.15	6.06	6.06
Acute nephritis	2.95	2.95	6.31	6.31
" "	3.10	3.10	6.12	6.12

TABLE II.

Miscellaneous.

Sample.	$\frac{M}{58.5}$ KI.		NaCl per cc. of sample.	
	New method.	McLean and Van Slyke.	New method.	McLean and Van Slyke.
	cc.	*cc.*	*mg.*	*mg.*
Pleural fluid	3.50	3.45	5.62	5.68
" "	3.20	3.20	6.00	6.00
Hydrocele fluid	3.10	3.10	6.12	6.12
Cerebrospinal fluid	2.20	2.20	7.25	7.25
Albuminous urine	5.25	5.25	3.42	3.42
" "	5.10	5.07	3.62	3.66
" "	1.55	1.55	8.06	8.06

It will be seen that the results obtained by the new method are in excellent agreement with those obtained by the original method of McLean and Van Slyke. The greatest variation is 0.05 cc. $\frac{M}{58}$ KI solution, an error identical with that given by McLean and Van Slyke, so that it is with the greatest confidence that we can recommend our alternative method to those laboratories which may find themselves inconvenienced by the present shortage of "Merck's blood charcoal reagent."

SUMMARY.

An alternative method has been devised for the removal of the blood proteins, previous to the estimation of the chlorides, by the method of McLean and Van Slyke, which does not require the use of a highly specialized reagent.

The method is just as easy, simple, and accurate as the original coagulation method. It is applicable to all the various body fluids.

INTRAVENOUS INJECTIONS OF β-HYDROXYBUTYRIC AND ACETOACETIC ACIDS.

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(Received for publication, May 14, 1917.)

β-Hydroxybutyric Acid.

The sodium salt of *l*,β-hydroxybutyric acid in 2 to 3 per cent solution was injected intravenously into normal, well nourished, resting dogs at selected rates maintained uniformly during periods of 2 hours. The urine excreted during the injections was examined for acetone, acetoacetic acid, and *l*,β-hydroxybutyric acid.

With injections of sodium *l*,β-hydroxybutyrate at the rate of 0.3 gm. (0.0024 gm. mol.) per kg. of body weight per hour, no optically active hydroxybutyric acid became demonstrable in the urine (one experiment). With the injection of 0.4 gm. (0.0032 gm. mol.) per kg. per hour, a trace of *l*,β-hydroxybutyric acid was excreted (one experiment); that is, in the case of a dog weighing 6.35 kg. receiving 2.54 gm. of the sodium salt per hour for 2 hours the rotation of the residue obtained by ether extraction of the total urine, when made up to 10 cc. and read in a 10 cm. tube, was $-0.08°$, corresponding to about 0.033 gm. of the acid. In an experiment with another animal 0.4 gm. of the sodium salt per kg. per hour caused no detectable excretion of levorotatory substance. With an injection of 0.5 gm. (0.0040 gm. mol.) per kg. per hour in the case of a 6.13 kg. dog, 0.050 gm. of *l*,β-hydroxybutyric acid was recovered in the urine. The sodium nitroprusside-ammonia tests for acetone and the Gerhardt ferric chloride reaction for acetoacetic acid proved negative in each of these urines.

These experiments indicate a normal intravenous tolerance limit[1] for sodium l,β-hydroxybutyrate at or about 0.0032 gm. mol. of the salt, or 0.0026 gm. mol. of the free acid per kg. of body weight per hour. The appearance of l,β-hydroxybutyric acid in the urine of a dog might therefore be taken to indicate that its rate of entry into the blood exceeds this value, provided the power of the organism to utilize β-hydroxybutyric acid is not diminished.

Acetoacetic Acid.

Experiments performed in an identical manner with the sodium salt of acetoacetic acid resulted as follows: Injections at the rate of 0.1 gm. (0.0008 gm. mol.) per kg. per hour did not lead to the appearance of acetone, acetoacetic acid, or β-hydroxybutyric acid in the urine (two experiments). With injections of 0.2 gm. (0.0016 gm. mol.) per kg. per hour, acetone and acetoacetic acid became demonstrable in the urine, but there was no excretion of β-hydroxybutyric acid (three experiments). An injection of 0.3 gm. (0.0024 gm. mol.) per kg. per hour, in one case, and of 0.4 gm. (0.0032 gm. mol.) per kg. per hour in another, also led to the excretion of traces of acetone and acetoacetic acid, but no l,β-hydroxybutyric acid. However, in the case of another dog receiving 0.4 gm. (0.0032 gm. mol.) per kg. per hour l,β-hydroxybutyric acid was detected in the urine, and following an injection of 0.5 gm. per kg. per hour in a dog weighing 5.1 kg., 0.041 gm. of l,β-hydroxybutyric acid appeared in the urine. With higher rates of injection of sodium acetoacetate proportionately greater amounts of l,β-hydroxybutyric acid were recovered.

[1] The rate at which a substance such as glucose or β-hydroxybutyric acid must be introduced into the blood from without in order to cause the substance to appear in the urine does not of necessity represent the rate at which the substance must enter the blood from all sources, endogenous as well as exogenous, to produce this effect. For during the intravenous injection of a substance at a rate a, the same substance may theoretically be entering the blood from endogenous sources at a rate x, so that the actual rate of entry which just suffices to cause the substance to appear unchanged in the urine would be $a + x$. The term *intravenous tolerance limit* is used simply to designate the rate of intravenous injection which just suffices to cause an overflow into the urine of the substance injected.

DISCUSSION.

In these experiments l,β-hydroxybutyric acid became demonstrable in the urine when acetoacetic acid was brought into the systemic venous blood at a rate of 0.0032 gm. mol. of the sodium salt per kg. per hour, or at the same rate at which the sodium salt of l,β-hydroxybutyric acid itself had to be injected by the same route in order to cause a demonstrable excretion of this acid. It would therefore appear that, in so far as the excretion of hydroxybutyric acid is concerned, the intravenous injection of sodium acetoacetate is equivalent to the injection of the same number of molecules of the former acid itself, which strongly suggests that acetoacetic acid when injected into the systemic blood stream under the conditions of the experiments described, *is reduced almost quantitatively into the hydroxy acid and at a velocity not less than that of the injection*, because were this not the case, the rate of formation of β-hydroxybutyric acid would not suffice to overstep the tolerance limit for this substance as above established.

Two objections might be raised against this interpretation: (*a*) During the injections of acetoacetic acid at the rates necessary to produce detectable excretions of l,β-hydroxybutyric acid in the urine, some acetoacetic acid was excreted as such and in addition some came out as acetone. However, the quantities so accounted for were too small to affect the argument. Thus a dog which weighed 12.5 kg. received in 2 hours an actual total of 10 gm. of sodium acetoacetate, and from one-half the total urine collected during the injection period there was recovered 0.027 gm. of acetone, corresponding to a total excretion for the period of 0.095 gm. of acetoacetic acid or about 1 per cent of the material injected. (*b*) There is the possibility that the observed excretions of β-hydroxybutyric acid coincident with injections of sodium acetoacetate might arise from indirect effects of the acetoacetate, such as a diminution of the rate of utilization of endogenous β-hydroxybutyric acid, an increased endogenous supply of the latter substance, or some unforeseen disturbance of metabolism. This possibility has not been fully excluded. A control was made with sodium butyrate administered at the rate of 0.75 gm. per kg. per hour for 1 hour and in this case no β-hydroxybutyric acid, acetoacetic acid, or acetone was found in the

urine. Moreover the administration of sodium acetoacetate at rates below 0.4 gm. per kg. per hour failed to cause any excretion of β-hydroxybutyric acid.

The experiments further directly suggest that during the injection of *l*,β-hydroxybutyric acid at rates up to 0.5 gm. per kg. per hour, less than 40 per cent at most of the hydroxy acid can be converted into acetoacetic acid because the tolerance limit for the latter substance, in the form of its sodium salt, lies below 0.2 gm. (0.0016 gm. mol.) per kg. per hour and injections of sodium β-hydroxybutyric acid at 0.5 gm. (0.0040 gm. mol.) per kg. per hour were not accompanied by any excretion of acetoacetic acid. There is indeed no direct evidence in these experiments that any of the injected hydroxybutyric acid was oxidized to acetoacetic acid and the evidence obtained by injections of acetoacetic acid indicates that if any of this acid were formed it would necessarily be reconverted into the hydroxy acid. It would appear therefore that if acetoacetic acid and hydroxybutyric acid are in equilibrium in the organism, as held by Neubauer and others, the balance in health is swung heavily toward the hydroxybutyric side.

In 1910 Blum[2] reported the excretion of *l*,β-hydroxybutyric acid in the urine following sufficiently large subcutaneous injections of the acetoacetic acid sodium salt, and almost simultaneously Dakin[3] reported similar results following intravenous injections as well. Marriott[4] was able to demonstrate an increase of the β-hydroxybutyric acid concentration in the blood and tissues and its excretion in the urine following intravenous administrations of sodium acetoacetate but was not successful in demonstrating the conversion of β-hydroxybutyric acid into acetoacetic acid. The results of the present experiments confirm these observations and add data of a quantitative character. Incidentally they serve to illustrate the use of the method of timed intravenous injections in a problem of intermediate metabolism.

In a later paper we hope to report on the relationships between acetoacetic and β-hydroxybutyric acids under other metabolic conditions.

[2] Blum, L., *Münch. med. Woch.*, 1910, lvii, 683.

[3] Dakin, H. D., *J. Biol. Chem.*, 1910, viii, 97.

[4] Marriott, W. M., *J. Biol. Chem.*, 1914, xviii, 241.

CONCLUSIONS.

I. *l*,β-Hydroxybutyric acid became demonstrable in the urine of normal dogs receiving continuous intravenous injections of sodium *l*,β-hydroxybutyrate at the rate of 0.4 gm. (0.0032 gm. mol.) per kg. of body weight per hour for 2 hours, but not with lower rates. Sodium acetoacetate appeared in the urine when injected for the same time at the rate of 0.2 gm. (0.0016 gm. mol.) per kg. per hour, but not with lower rates.

II. Sodium acetoacetate when injected at rates close to 0.4 gm. (0.0032 gm. mol.) per kg. per hour gave rise to the excretion in the urine of *l*,β-hydroxybutyric acid.

III. Since the number of molecules of sodium acetoacetate required per kg. of body weight per hour to cause the appearance of *l*,β-hydroxybutyric acid in the urine did not differ appreciably from the number of molecules of the sodium salt of *l*,β-hydroxybutyric acid itself which were necessary to produce the same effect, it would appear most probable that acetoacetic acid, under the conditions of these experiments, is converted almost quantitatively into *l*,β-hydroxybutyric acid.

IV. The experiments afford no direct evidence to indicate that any of the injected *l*,β-hydroxybutyric acid was oxidized to acetoacetic acid.

EXPERIMENTAL.

Material.—*l*,β-Hydroxybutyric acid was obtained by ether extraction of diabetic urine which had been concentrated by evaporation. The calcium zinc salt of the acid was made from this extract by Shaffer's method[5] and purified by recrystallization. From the pure salt the free acid was obtained by acidifying with phosphoric acid and ether extraction. The residue from the ether was dissolved in water and the acid determined by titration and polariscope. The entire amount was neutralized with sodium carbonate. Sodium chloride and water were then added to give solutions for injection containing from 2 to 3 per cent sodium *l*,β-hydroxybutyric acid and 0.5 per cent sodium chloride.

[5] Shaffer, P. A., and Marriott, W. M., *J. Biol. Chem.*, 1913–14, xvi, 265.

Acetoacetic acid was prepared from its ethyl ester after the method of Ceresole.[6] The ester was saponified, the solution then saturated with ammonium sulfate, and extracted with ether. The ether residue was neutralized with barium carbonate and the unchanged ester removed by extraction with ether. The acetoacetic acid was then freed with sulfuric acid and extracted with ether. The ether residue was dissolved in water and the acid content of this solution determined by titration and as acetone. Sodium carbonate, in amount just sufficient to neutralize, and sodium chloride and water were added so that the solutions for injection contained from 2 to 5 per cent of sodium acetoacetate and 0.5 per cent of sodium chloride. The acetoacetic acid was freshly prepared for each experiment.

Animals.—Uniform injection rates were secured by means of a volumetric pump described by Woodyatt.[7] The volumes actually injected in the various experiments ranged from 31 to 124 cc. per hour and never deviated more than 1 or 2 per cent from the volume which it was desired to give. There was no difficulty in maintaining uniform rates. In each experiment the following procedure was adhered to: The dog was made as comfortable as possible on a padded animal board and a superficial vein of the leg was exposed under 0.5 per cent cocaine. An hour later the bladder was emptied by catheter and a 2 hour fore-period commenced. At the end of this period a cannula was gently slipped into the vein, the bladder emptied and irrigated, and the injection started. After 2 hours the bladder was again emptied and the injection stopped. In some cases urine was collected for an after-period of 2 hours, the dog remaining on the table.

Analyses.—The urines were examined for acetone by the sodium nitroprusside-ammonia test and for acetoacetic acid by the Gerhardt ferric chloride reaction. Free acetone was determined by aeration into standard iodine by Folin's method, and acetoacetic acid was titrated as acetone, after distillation, by a conventional iodine thiosulfate method. *l*,β-Hydroxybutyric acid was determined by ether extraction and polariscope according to Bergell.

[6] Ceresole, *Ber. chem. Ges.*, 1882, xv, 1327.

[7] Woodyatt, R. T., *J. Biol. Chem.*, 1917, xxix, 355.

TABLE I.

Intravenous Injections of Sodium l,β-Hydroxybutyrate.

Experiment.	Date.	Dog.	Body weight.	Injection. Rate per kg. per hour.	Injection. Duration.	Injection. Total injected.	Urine findings. Nitroprusside test.	Urine findings. Gerhardt test.	Urine findings. l,β-Hydroxybutyric acid.
	1917		*kg.*	*gm.*	*hrs.*	*gm.*			*gm.*
1	March 9	A.	6.60	0.30	2	3.96	0	0	0
2	" 15	"	6.35	0.40	2	5.09	0	0	0.033
3	" 27	B.	5.12	0.40	2	4.10	0	0	0
4	" 21	A.	6.13	0.50	2	6.13	0	0	0.050

TABLE II.

Intravenous Injections of Sodium Acetoacetate.

Experiment.	Date.	Dog.	Body weight.	Injection. Rate per kg. per hour.	Injection. Duration.	Injection. Total injected.	Urine findings. Nitroprusside test.	Urine findings. Gerhardt test.	Urine findings. Free acetone.	Urine findings. Acetone and acetoacetic acid.*	Urine findings. l,β-Hydroxybutyric acid.
			kg.	*gm.*	*hrs.*	*gm.*			*gm.*	*gm.*	*gm.*
5	May 11, 1916	C.	9.70	0.10	2.0	1.94	0	0	—	—	—
				0.20	0.5	3.88	+	+	—	—	—
6	April 18, 1917	D.	11.10	0.10	2.0	2.22	0	0	—	0.005	—
				0.20	2.0	4.44	+	+	—	0.015	—
7	" 18, 1917	E.	13.40	0.30	2.0	8.04	+	+	0.006	0.033	0
8	" 8, 1917	F.	12.50	0.40	2.0	10.00	+	+	—	0.095	0
9	June 1, 1916	G.	9.66	0.40	2.0	7.72	+	+	—	—	0.028
10	April 8, 1917	B.	5.10	0.50	2.0	5.10	+	+	—	—	0.041
11	May 25, 1916	C.	9.55	0.70	3.0	13.75	++	++	—	0.547	0.414
12	June 21, 1916	H.	8.63	0.85	2.0	14.68	++	++	—	—	0.514

* Calculated in terms of acetoacetic acid.

THE QUANTITATIVE ESTIMATION OF DEXTROSE IN MUSCULAR TISSUE.

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INTRODUCTION.

The need for an accurate and convenient method for the determination of dextrose in muscular tissue is well known to those who have had occasion to do work along that line. The importance of having such a method can hardly be overestimated. In fact, it may be said that the solution of one of the most important problems which has baffled physiological chemists for a long time—the behavior of sugar in the animal body—depends in no small measure upon the accurate estimation of dextrose in muscular tissue.

The problem is extremely simple in principle. It consists essentially in the removal from a water extract of the tissue of all constituents which interfere with the normal reducing action of dextrose upon a hot alkaline copper solution. When this has been accomplished the dextrose may be determined by any one of several reduction methods. The constituents of a water extract of muscular tissue which prevent the normal action of dextrose upon a hot alkaline copper solution are of two classes: (1) those constituents which retard or prevent the precipitation of copper by dextrose; (2) those constituents which either of themselves reduce the copper solution or cause the dextrose to reduce an abnormal quantity. The substances of the first class may be readily removed by several reagents commonly used for the precipitation of proteins. The difficulty lies in removing the substances of the second class. Such reagents as neutral and basic lead acetate and picric acid are entirely inefficient for this purpose. The use of mercuric salts, either

singly or in combination with phosphotungstic acid, seems to have met with some success. It does not appear that phosphotungstic acid has been successfully used alone.

It does not seem necessary to give an extended discussion of the various methods which have been proposed for the determination of dextrose in muscular tissue. Experience has proven many of them to be clearly inefficient. There are a few methods, however, which seem to have given satisfaction in the hands of their authors.

Previous Methods.

Storp (1) developed a method in which a combination of mercuric acetate and phosphotungstic acid were used as clarifying reagents. Dextrose was determined by Bertrand's method. It was found, however, that when a water extract of muscular tissue was fermented with yeast so as to destroy all dextrose present and then clarified by the author's method, the alkaline copper solution was still reduced to an appreciable degree. Calculated in terms of dextrose in the tissue, the reduction varied from 0.015 to 0.06 per cent. The author did not consider that this error appreciably affected the accuracy of his method. However, when one is dealing with quantities of dextrose as low as 0.10 to 0.15 per cent, such as may occur in muscular tissue immediately after the death of the animal, the error seems too large to be disregarded. In addition, the use of a mercuric salt, with the necessity for its subsequent removal by means of hydrogen sulfide, makes the method rather cumbersome and time consuming.

Smith (2) studied the use of mercuric acetate, mercuric nitrate, and a combination of picric and phosphotungstic acids as clarifying reagents for the determination of sugar in meat products, particularly meat extract. Dextrose was determined by reduction of Fehling's solution, and the reduced copper was estimated by Low's iodide method. While the use of mercuric salts under proper conditions yielded satisfactory results, yet a combination of picric and phosphotungstic acids gave equally good results. The latter reagents are recommended on account of the greater convenience attending their use. Most of the work reported deals with the determination of sugar in meat extracts and cured meats rather than fresh muscular tissue. It is stated that in the presence of sucrose dextrose may be determined within an error of 0.10 to 0.20 per cent, and that total dextrose may be determined within an error of 0.10 per cent.

In principle, the method is applicable to the determination of dextrose in muscular tissue, but the details which are given for the estimation of sugar in meats would have to be modified. The quantity of material recommended—50 gm. of meat to a volume of 250 cc.—is too small, and corresponding changes would have to be made in the quantities of clarifying reagents.

EXPERIMENTAL.

These experiments were undertaken with two objects in view: (1) to identify the copper-reducing substances, other than dextrose, frequently present in the water extracts of muscular tissue clarified by various reagents; (2) to remove those substances from solution in as simple a manner as possible, together with other substances which interfere with the normal reducing action of dextrose upon Fehling's solution.

If a water extract of muscular tissue, which has been concentrated to a suitable volume and clarified by means of neutral or basic lead acetate, is boiled with Fehling's solution the reduction will in all probability be abnormal. The boiling Fehling's solution is apt to be muddy green in appearance and the copper precipitate more or less yellow in color and perhaps flocculent in character. A considerable part of the yellow copper precipitate will go through the Gooch crucible on filtration and washing. Similar conditions are frequently observed when water extracts of muscular tissue which have been imperfectly clarified by means of other reagents, such as mercuric salts and phosphotungstic acid, are boiled with Fehling's solution.

A general survey of the water-soluble constituents of muscular tissue not readily precipitated by the reagents commonly used for the clarification of such solution for the determination of dextrose, and which reduce Fehling's solution, led to the conclusion that in all probability the interfering substances were creatine and creatinine, the latter in particular. The reducing action of these compounds upon boiling Fehling's solution is well known. Abderhalden (3) states that creatine reduces Fehling's solution without the separation of cuprous oxide, and that creatinine reduces the copper solution on continued boiling. On boiling for $\frac{1}{2}$ hour one molecule of creatinine reduced three-fourths of a molecule of CuO. Cuprous oxide was not formed. Similar information regarding the copper-reducing properties of creatine and creatinine may be found in various texts.

In order to test the action of creatinine upon Fehling's solution the following experiments were carried on. The Fehling's solution used in the work reported in this paper was that prepared according to the method of Allihn, and unless otherwise stated

reduction was carried on under the condition described for that solution.

0.05 gm. of creatinine in solution was added to boiling Fehling's solution. Reduction started after boiling for $1\frac{1}{2}$ minutes, and at the end of 2 minutes the solution had turned muddy yellow in color. There was considerable reduced copper. On filtration, a portion of the precipitate, which was bright red in color, remained in the Gooch crucible, while considerable yellow copper precipitate passed through the filter, particularly on washing. The reduced copper retained in the crucible weighed 0.0766 gm. The appearance of the Fehling's solution during reduction and of the reduced copper were very similar to conditions observed when imperfectly clarified extracts of muscular tissue are boiled with Fehling's solution.

0.1 gm. of creatinine in solution was added to boiling Fehling's solution. Reduction started after boiling $1\frac{1}{4}$ minutes when the solution turned muddy green in color; at the end of 3 minutes the solution was very muddy in appearance and reddish yellow in color; at the end of 4 minutes the solution was dark reddish brown in color. On filtration most of the reduced copper, which was reddish brown in color, remained on the filter. The reduced copper weighed 0.1776 gm.

In order to test the reducing action of creatinine upon Fehling's solution in the presence of dextrose the following tests were conducted:

0.05 gm. of creatinine and 0.1500 gm. of dextrose were added to boiling Fehling's solution. The solution first turned muddy green, then yellow, and finally reddish brown in color. The reduced copper was yellowish brown in color. It weighed 0.4036 gm., corresponding to 0.1889 gm. of dextrose, an increase of 0.0389 gm., or 23.9 per cent over the amount of dextrose added.

0.025 gm. of creatinine was added to Fehling's solution together with 0.125 gm. of dextrose. Reduction appeared to be practically normal. The reduced copper was red in color. It corresponded to 0.1483 gm. of dextrose, an increase of 0.0233 gm. or 18.6 per cent over the amount of dextrose added.

The results of the above tests show that creatinine has a very considerable reducing action upon Fehling's solution, whether used alone or in the presence of dextrose. It is apparent that the elimination of creatinine is necessary for the accurate determination of dextrose in muscular tissue by copper reduction methods.

A study of the reducing action of creatine upon Fehling's solution yielded the following results:

0.05 gm. of creatine was added to boiling Fehling's solution. Toward the end of the boiling period there appeared to be a slight reduction. On filtration there was a very small amount of yellowish red reduced copper on the filter.

0.075 gm. of creatine was added to boiling Fehling's solution. Toward the end of the boiling period the solution turned slightly greenish yellow in color. On filtration a very small amount of yellow reduced copper remained on the filter. On washing, this passed through the filter.

0.1 gm. of creatine was boiled with Fehling's solution with practically the same results as with the previous sample.

The following tests were carried on to determine the effect of creatine upon the reducing action of dextrose on Fehling's solution:

0.1 gm. of dextrose and 0.050 gm. of creatine were added to boiling Fehling's solution. The reduction appeared normal. The reduced copper corresponded to 0.1075 gm. of dextrose, an increase of 7.5 per cent over the amount of dextrose added.

0.1 gm. of dextrose and 0.075 gm. of creatine were boiled with Fehling's solution. The reduction appeared normal. The reduced copper corresponded to 0.1099 gm. of dextrose, an increase of 9.9 per cent over the amount added.

0.1 gm. of dextrose and 0.100 gm. of creatine were added to boiling Fehling's solution. The reduction appeared normal. The reduced copper corresponded to 0.1015 gm. of dextrose, an increase of 1.5 per cent over the amount added.

On the whole, these results show that creatine has a very slight reducing action on Fehling's solution, but that it slightly increases the reducing action of dextrose upon that solution. Later, experiments will be reported which were conducted to determine the effect of the creatine of muscular tissue upon the determination of dextrose in that tissue.

Since creatinine reduces Fehling's solution to a considerable degree, and creatine reduces the copper solution to a slight extent, it is apparent that if dextrose is to be accurately determined in muscular tissue by one of the copper reduction methods, creatinine must be removed from solution, and creatine probably ought to be removed. The problem is to remove these compounds, as well as all other substances which interfere with the accurate determination of dextrose in a water extract of muscular tissue, in as simple a manner as possible. The use of a single clarifying reagent is to be preferred.

According to Abderhalden (3) creatinine is precipitated by the following reagents: silver nitrate in the presence of ammonia, mercuric chloride, mercuric nitrate, phosphotungstic acid, and phosphomolybdic acid. Creatine is precipitated by mercuric nitrate but not by phosphomolybdic acid or lead acetate.

Since phosphotungstic acid is not only a highly efficient reagent for the precipitation of proteins, but also for most of the diamino and a part of the monoamino-acids as well, it was decided to attempt to develop a method in which phosphotungstic alone would serve as the clarifying reagent. While creatine is not precipitated by phosphotungstic acid, yet it is so readily converted into creatinine that it may be easily removed from solution by the same reagent.

It does not seem necessary to describe the preliminary experiments which were carried on before a successful method was developed for the determination of dextrose in muscular tissue. It may be said, however, that a very considerable amount of preliminary work was done. The method which is to be described is simple and has a high degree of accuracy. It has been used by the writer for 6 months in a study of the dextrose content of a very considerable number of samples of both fresh and autolyzed muscular tissue with highly satisfactory results. The dextrose content of the samples has varied from 0.03 to 0.49 per cent. The method is as follows.

Weigh 100 gm. of finely ground muscular tissue, previously freed from visible fat and connective tissue, into a 600 cc. beaker. Add 200 cc. of distilled water, gradually heat to boiling, and boil for a few minutes. The contents of the beaker must be stirred frequently during this and subsequent extractions. Remove the beaker from the flame, let stand a few minutes for the insoluble material to settle, and decant the clear liquid on to a previously prepared asbestos filter in a 4 inch funnel. Filter with the aid of suction. Add 150 cc. of hot distilled water to the residue in the beaker, boil a few minutes, let settle, decant the clear liquid, and filter as above. Repeat the operation and finally transfer the contents of the beaker to the funnel. Wash the residue on the filter with a little hot water and filter as dry as possible. Transfer the contents of the filter flask to an 800 cc. beaker, place it on a steam bath, and concentrate the liquid to a volume of about 25 to 30 cc. Do not allow the liquid to evaporate to dryness. Transfer the contents of the beaker to a 100 cc. volumetric flask, but do not allow the volume of the liquid to exceed 60 to 70 cc. Cool to room temperature, add 25 to 30 gm. of phosphotungstic acid dissolved in

about 25 cc. of water, shake vigorously, and let stand a short time for gas bubbles to rise to the surface. Make the solution to volume, shake, and either filter or centrifuge to remove solid matter. The use of a centrifuge is preferable since a much larger volume of filtrate is obtained than when the material is filtered through filter paper. Filtration through a dry asbestos mat with the aid of suction is also a desirable method. Test a portion of the filtrate for complete precipitation by the addition of dry phosphotungstic acid. In case an appreciable precipitate forms, take an aliquot portion of the filtrate, add an excess of dry phosphotungstic acid, make to volume, filter, and test the filtrate for complete precipitation. If precipitation is complete, add sufficient dry potassium chloride to the filtrate to precipitate the excess of phosphotungstic acid. One part of phosphotungstic acid requires 0.076 parts of potassium chloride for complete precipitation in an acid solution. Filter off the potassium phosphotungstate precipitate and test the filtrate for the presence of creatinine. When an appreciable excess of phosphotungstic acid has been used for clarification, not more than a trace of creatinine will ordinarily be found. Determine the reducing action of duplicate aliquot portions of 25 cc. of the filtrate by Allihn's method and estimate the reduced copper by Low's iodide method.[1]

Certain precautions must be observed in order to secure accurate results with the above method. There must be an appreciable excess of phosphotungstic acid as shown by the addition of that acid to the clarified filtrate, and also by the formation of an appreciable precipitate on the addition of potassium chloride. The excess of phosphotungstic acid must be removed since it interferes with the normal reducing action of dextrose on Fehling's solution. Not more than a trace of creatinine should be present in the clarified extract. The clarified solution should be boiled with Fehling's solution without delay so as to avoid any subsequent conversion of creatine into creatinine. The use of

[1]Since submitting this manuscript for publication the author's attention has been called to an article by Palmer (*J. Biol. Chem.*, 1917, xxx, 79) in which he reports a considerable formation of dextrose in muscular tissue during grinding for analysis. To guard against this change Palmer plunges the fresh tissue into boiling water, and after boiling for some time decants the clear liquid and macerates the tissue. In view of Palmer's findings it is suggested that, when the dextrose content of muscular tissue is to be determined immediately after the death of the animal, the weighed quantity of tissue be cut into several pieces, plunged into boiling water, and the boiling continued for 5 to 10 minutes. Decant and filter the clear liquid, grind the tissue in a meat grinder, and proceed with the extraction as directed.

hydrochloric or sulfuric acid in conjunction with phosphotungstic acid is not necessary.

Table I shows results obtained when known amounts of dextrose were added to muscular tissue and the dextrose was determined by the method described.

TABLE I.

Sample No.	Dextrose.				Error.
	In muscular tissue.	Added.	Calculated.	Found.	
	per cent	*per cent*	*per cent*	*per cent*	
1	0.274			0.274	
2	0.274	1.00	1.274	1.298	+0.026
3	0.274	1.50	1.774	1.780	+0.006
4	0.274	2.00	2.274	2.290	+0.016
5	0.322			0.322	
6	0.322	0.50	0.822	0.766	−0.056
7	0.322	1.25	1.572	1.556	−0.016
8	0.322	2.50	2.822	2.860	+0.038
9	0.307			0.307	
10	0.307	0.37	0.677	0.676	−0.001
11	0.307	0.69	0.997	1.031	+0.034
12	0.307	1.16	1.467	1.411	−0.056

Table II shows results obtained when dextrose was determined in four portions of the same sample of muscular tissue.

TABLE II.

Sample No.	Dextrose.	Remarks.
	per cent	
1	0.262	
2	0.265	
3	0.265	Extract evaporated in presence of $CaCO_3$.
4	0.254	

The reducing action of creatine upon Fehling's solution, either singly or in the presence of dextrose, has been discussed. Since creatine is not precipitated by phosphotungstic acid, the question arises as to what effect the presence of creatine has upon the determination of dextrose in muscular tissue by the method

described by the writer. In order to get the desired information dextrose was determined in muscular tissue both with and without the removal of creatine from a water extract of the tissue. Creatine is readily removed from a water extract of muscular tissue by the following simple modification of the method described for the determination of dextrose.

Take 25 cc. of the filtrate from the phosphotungstic acid precipitation from which the excess of that reagent has not been removed, transfer to a 50 cc. volumetric flask, add 2.5 cc. of concentrated HCl, and heat in an autoclave for 15 minutes at 240°F. to convert creatine into creatinine. Cool the contents of the flask to room temperature. If an appreciable quantity of phosphotungstic acid is present the creatinine will be precipitated; if not, add about 5 gm. of phosphotungstic acid, make to volume, and filter. Add sufficient dry KCl to the filtrate to precipitate the excess of phosphotungstic acid, filter, and determine the dextrose in the filtrate. Test the filtrate for the presence of creatinine.

Table III shows the results obtained in the determination of dextrose in muscular tissue with and without the removal of creatine.

TABLE III.

Sample No.	Dextrose by regular method.	Dextrose, creatine removed.	Dextrose added to tissue.
	per cent	*per cent*	*per cent*
1	0.307	0.305	None.
2	0.676	0.684	0.370
3	1.031	1.054	0.690
4	1.411	1.480	1.160

On the whole, these results show a slightly larger percentage of dextrose recovered after the removal of creatine than before. The contrary was to have been expected, and since the differences are comparatively small, it may be considered that the presence of creatine does not introduce an appreciable error into the determination of dextrose by the method described by the writer; hence its removal does not appear necessary.

As has been previously noted, Storp found that a water extract of muscular tissue which had been fermented with yeast and clarified by means of phosphotungstic acid and mercuric acetate still reduced Fehling's solution to an appreciable degree. In

order to determine whether there are any copper-reducing substances, other than dextrose, present in muscle extract clarified by the use of phosphotungstic acid as described by the writer, the following experiment was conducted.

200 cc. of sterile plain beef broth, representing 100 gm. of beef, were inoculated with a culture of *Bacillus coli* and incubated for 42 hours at 37°C. in order to destroy muscle sugar. The broth was then transferred to a beaker and concentrated to the desired volume on a steam bath when dextrose was determined by the described method. On boiling with Fehling's solution only a mere trace of copper was reduced, not sufficient to be determined quantitatively. It must be considered also that creatine had not been removed from the clarified extract.

The extent to which nitrogenous compounds are removed from solution by the use of phosphotungstic acid in the clarification of a water extract of muscular tissue for the determination of dextrose may be of interest. In the case of one sample examined it was found that the clarified filtrate contained only 7.7 per cent of the nitrogen originally present in the hot water extract of the tissue, 92.3 per cent of the nitrogen having been precipitated. With a sample of commercial beef extract 90.66 per cent of the nitrogen originally present was precipitated on clarification by the same method.

Neuberg and Ishida (4) found that the use of a combination of mercuric acetate and phosphotungstic acid caused the precipitation of 92 per cent of the total nitrogen of a solution of hydrolyzed proteins.

Smith (5) found that with the method which he employed for the determination of sugar in meat extracts, phosphotungstic and picric acids being used as clarifying agents, about five-sixths (83 per cent) of the nitrogen was precipitated.

SUMMARY.

On account of its reducing action on Fehling's solution, creatinine is an important source of error in the determination of dextrose in muscular tissue. Creatinine is precipitated by an excess of phosphotungstic acid, and since that reagent is also an efficient precipitant for other nitrogenous constituents of muscular tissue as well, the use of phosphotungstic acid in excess has

proven to be an excellent method for the clarification of a water extract of muscular tissue for the determination of dextrose by means of Fehling's solution. A method for the determination of dextrose in muscular tissue based on these principles has been described. This method has proven to be simple in operation, and has yielded accurate results.

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THE BLOOD LIPOIDS IN ANEMIA.

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The pathogenesis of anemia and particularly of pernicious anemia is a subject that has stimulated interest and investigation for many years but so far without definite results although many theories have been advanced to account for the conditions. The present opinion seems to be that the phenomena as observed are manifestations of a primary disturbance the nature of which is still unknown. Since a characteristic feature of the disease is the destruction of the red blood cells, much attention and investigative work has been directed to hemolytic and antihemolytic substances and particularly to various lipoids.

Berger and Tsuchiya (1) reported that the ether extract of the intestinal mucosa of a patient dead with pernicious anemia had several times greater hemolytic power than that from normal persons. Later work by McPhedran (2) failed, however, to substantiate this difference as characteristic. Faust and Tallquist (3) found hemolytic lipoids in the pancreas and gastrointestinal mucous membrane of persons not suffering from anemia. Kullmann (4) and later Faust and Tallquist (3) found that the lipoids of cancer tissue were hemolytic.

The most suggestive work regarding the rôle of lipoids in anemia has been done in connection with the anemia produced by the broad tapeworm, *Botriocephalus latus*. As early as 1888 Schapiro (5) described a type of anemia very similar to pernicious anemia which he believed to be produced by this worm since the symptoms ceased when it was removed. Tallquist (6) demonstrated the presence of hemolytic lipoids in the worm and later with Faust (3) isolated and described the hemolytic substance which they found to be cholesterol oleate. Faust and Tallquist found further that the hemolytic properties were entirely due to the oleic acid which this substance contained and also that a strongly hemolytic chyle, owing its hemolytic properties to abnormal amounts of sodium oleate, could be obtained in dogs by feeding oleic acid. Faust (7) found that long continued administration of oleic acid, either by mouth or subcu-

taneously, to dogs and rabbits produced anemic conditions. Adler (8) by similar feeding of non-toxic amounts of olive or cottonseed oil was able to produce in rabbits blood crises resembling those of pernicious anemia. Since the hemolytic agent in all these experiments was obviously the unsaturated fatty acids, the results indicate that under certain conditions toxic quantities of these acids or their (toxic) derivatives may reach the blood by way of the chyle—a fact which heretofore has not been realized. The *Botriocephalus* anemia has been explained (3) as due to abnormal absorption resulting from intestinal changes brought about by the worm.

Three possible explanations are available to account for the anemic conditions produced by long continued feeding of unsaturated fats: (1) an abnormality of the absorptive mechanism, allowing abnormal amounts of hemolytic lipoids to reach the blood; (2) a failure of the assimilative mechanism in the blood or tissues resulting in an abnormal accumulation of these substances either free or in the form of toxic derivatives; or (3) a decrease in the antihemolytic substances in the blood. That changes in the unsaturated fatty acids may be produced during normal absorption has been shown to be probable by results obtained during a study of fat absorption (9). When olive oil was fed to dogs it was found that the fatty acids of the chyle fat had a lower iodine number and a higher melting point than those of the oil fed and a considerable fraction of saturated fatty acids could be separated. The conclusion drawn at the time was that there had been an admixture of saturated fat from some other source—possibly the liver—but the results could be equally well explained as due to the reduction of a portion of the unsaturated acids during absorption—a mechanism for regulating the amount of unsaturated fatty acids absorbed. The possibility may well therefore be kept in mind that a disturbance of absorption or assimilation of the unsaturated fats may be responsible for human anemia. The idea is familiar enough that many bodily processes, notwithstanding a considerable "factor of safety," are liable to fail when continuously overworked; also that the inherent capacity of a mechanism varies in different individuals. The anemic individual may be unable to metabolize normal amounts of unsaturated fatty acids just as the diabetic for similar reasons is unable to utilize normal amounts of dextrose.

Oleic acid being an unsaturated fatty acid, the hemolysis produced by it was ascribed to the presence of the double bond and the inference was that the more double bonds the greater would be the hemolytic power.

Lamar (10) working with the pneumococcus actually found that the intensity of the lytic action of various unsaturated fatty acids was proportional to the degree of unsaturation. On the other hand McPhedran (2) in testing the hemolytic activity of these acids found no such proportionality and concluded that there was no evidence of the presence in the human body at any time of fatty acids appreciably more hemolytic than oleic acid. The only antihemolytic substance so far investigated has been cholesterol. It is a natural constituent of the plasma and corpuscles and its protective power against certain hemolytic agents (saponin) is well known. Since it is absorbed from the intestine with considerable readiness its therapeutic use in anemia seemed a logical procedure but the results of the treatment do not appear to have been satisfactory. Reicher (11) found improvement in a number of cases of anemia after feeding cholesterol while Klemperer (12) had very little success with the treatment.

Although cholesterol and lecithin have been shown to act antagonistically in certain types of hemolysis (*e.g.*, by cobra venom (13)) and both are believed to take an active part in fat metabolism (14) a disturbance of which has been indicated as a possible factor in the production of anemia, very little attempt has been made to make a comprehensive study of these substances or of the fat of the blood in anemia.

Medak (15) stimulated by the work of Faust and Tallquist made analyses of the blood lipoids in two cases of pernicious anemia and found that the percentage of cholesterol esters was abnormally high and also that the iodine value of the combined fatty acids was enormously increased above the normal values. King (16) in three cases of pernicious anemia found the total lipoids abnormally high while the free cholesterol was below normal. He also found that the iodine values of the fatty acids were often very high and he believed that there was a relation between these high values and the severity of the anemia. Some of the iodine values reported by King and Medak are impossibly high and they have been shown by Csonka (17) to be the result of incorrect calculations. Csonka reported iodine values for the blood fat of three cases of pernicious anemia which are probably not much, if any, above the normal although he gives no normal values for comparison.

In view of the small amount of available information regarding the lipoids of the blood in anemia it seemed desirable to under-

take a study of the amounts and relations of these substances in the blood in order to determine to what extent if any they were abnormal and to relate the data so obtained to the present knowledge both of anemia and of fat metabolism.

The cases studied were hospital patients, mostly suffering from pernicious anemia with, however, a few cases of secondary anemia including one from *Botriocephalus latus*. In most instances blood samples were taken more than once in order to follow the course of the disease or to determine the effect of treatment. The blood was taken in all cases before breakfast and was worked up as promptly as possible to avoid changes taking place after the blood was drawn. Sodium citrate (two drops of saturated solution per 10 cc. of blood) was used to prevent coagulation. Samples for analysis were taken of the well mixed whole blood and after centrifugation (10 minutes at 3,800 R.P.M.) of the plasma. The centrifugation was carried out in graduated tubes and the percentage of corpuscles calculated. From this number and the lipoid values for whole blood and plasma the lipoid composition of the corpuscles was computed. The methods for determining the blood lipoids have been described (18, 19) and need not be given here. Direct determinations were made of total fat (which includes total fatty acids and cholesterol), lecithin, cholesterol, and, in the later part of the work, of cholesterol esters. 10 cc. of the alcohol-ether extract were used for total fat, 15 to 25 cc. for lecithin, 10 to 20 cc. for cholesterol, and 25 to 30 cc. for cholesterol esters. Hemoglobin was determined by the method of Sahli and the corpuscle counts were made in the regular way. The value total fatty acids in the table was obtained by subtracting the value for cholesterol from the value for total fat. It represents the fatty acids in whatever combination they are present in blood—as fat, lecithin, cholesterol esters, soaps, etc. The value fat represents all the fatty acids not combined as lecithin or as cholesterol esters. It is assumed that they are present in combination with glycerol as ordinary fats but any fatty acid present in the blood, free, as soaps, or in any other form of combination, would be included in this value. The calculations for fat are based on the assumption that 0.7 of the lecithin and 0.4 of the cholesterol esters are

fatty acids and that 0.6 of the cholesterol in the plasma and none in the corpuscles is combined as esters. The value lecithin includes not only ordinary lecithin but also cephalin and other forms of "organic" phosphorus which are soluble in alcohol-ether. No water-soluble phosphorus has ever been found to be present in these blood extracts. The value cholesterol is total cholesterol including both the free and that in combination as ester. The value total lipoids is obtained by adding together the values for fat, lecithin, cholesterol, and cholesterol esters, and is approximately equivalent and comparable with the total fat or total ether extract of the earlier investigators. The ratio lecithin: cholesterol is included in the table because of the antagonistic relationship in hemolysis which has been shown by these two substances. The ratio total fatty acids: lecithin is given because it gives information regarding the fat-assimilative power of the blood. When the value is high it is believed to indicate a deficient fat assimilation. The results of the determinations (expressed in gm. per 100 cc.) are given in Table I and, for convenience in comparison, the average values for normal individuals are included at the head of the table. These and other results given below are discussed together at the end of the paper.

The spleen is generally regarded as the organ where the destruction of the red blood cells takes place, and on the assumption that anemia is due to hyperactivity of the spleen its removal has been somewhat widely advocated. The resulting benefits are generally doubtful although there appears to be a temporary improvement (20). Some determinations on the blood lipoids in anemia before and after splenectomy have been reported by King (16). He found that the total lipoids increased after the operation both in plasma and corpuscles, also that the cholesterol in the plasma increased at the expense of the corpuscles. Having obtained from the Massachusetts General Hospital, through the kindness of Dr. W. Denis, samples of blood from anemia patients before and after splenectomy, analyses were made and the results are given in Table II. Along with these are included analyses of samples from two patients not suffering from anemia but whose spleens were removed for other reasons.

TABLE

Blood Lipoids

Case.	Disease.	Date.	Hemoglobin	Erythrocytes.	Corpuscles	Total fatty: Whole blood	Total fatty: Plasma.
			per cent		*per cent*		
Men.....	Average normal........	......	...	....	43	0.36	0.38
Women.....	" " ...	....	...	....	38	0.36	0.40
I M. A.	Pernicious anemia.	Dec. 7, 1915	42	1,800,000	17	0.41	0.44
		" 17, 1915	47	2,136,000	18	0.29	0.29
		" 27, 1915	45	1,264,000	20	0.35	0.36
		Jan. 21, 1916	59	2,202,000	28	0.41	0.42
II P. B.	" "	Mar. 6, 1916	25	980,000	10	0.37	0.37
		" 31, 1916	26	1,384,000	9	0.41	0.41
III A. D.	" "	Jan. 30, 1916	42	1,400,000	16	0.44	0.46
		Mar. 6, 1916	16	700,000	9	0.39	0.39
		" 31, 1916	38	1,480,000	18	0.40	0.42
IV M. C.	" "	Feb. 23, 1916	25	1,040,000	11	0.49	0.51
		Mar. 6, 1916	42	1,400,000	14	0.39	0.39
		" 31, 1916	42	2,136,000	16	0.36	0.36
V F. B.	" "	Jan. 31, 1916	47	1,350,000	21	0.48	0.53
		Feb. 4, 1916	48	1,500,000	18	0.60	0.64
VI M. G.	Secondary "	Jan. 29, 1916	40	4,728,000	30	0.41	0.41
VII C. S.	" "	" 29, 1916	55	3,368,000	21	0.45	0.50
VIII L. H	" "	" 29, 1916	45	2,696,000	22	0.43	0.45
IX E. T.	Pernicious "	Dec. 13, 1915	60	2,900,000	25	0.44	0.41
		" 18, 1915	50	1,900,000	16	0.40	0.38
		" 27, 1915	25	1,248,000	13	0.39	0.38
X T. H.	" " (?)	Jan. 28, 1916	83	3,800,000	44	0.44	0.48
		Feb. 4, 1916	126	5,500,000	43	0.43	0.46
XI A. F.	*Botriocephalus.*	Dec. 2, 1915	38	1,340,000	12	0.43	0.44
		" 13, 1915	52	1,900,000	19	0.40	0.43
		" 18, 1915	51	1,900,000	20	0.42	
		" 27, 1915	80	2,272,000	30	0.40	0.37
XII E. S.	Lymphatic leukemia.	Feb. 21, 1916	81	3,260,000	32	0.62	0.62
XIII M. D.	Secondary anemia; gastric ulcer.	Dec. 18, 1915	58	2,900,000	24	0.41	0.39
XIV A. T.	Secondary anemia; internal hemorrhoids.	Jan. 22, 1916	50	3,118,000	24	0.43	0.44
XV A. B.	Carcinoma.	Nov. 8, 1915	84		34	0.40	0.44
XVI J. F.	"	Oct. 30, 1915	47	2,200,000	23	0.51	0.58

I.

ı Anemia.

;ids.	Lecithin.			Cholesterol.			Fat.		Total fatty acids. / Lecithin.			Lecithin. / Cholesterol.			Total lipoid.
Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Plasma.
0.35	0.30	0.22	0.40	0.21	0.22	0.19	0.14	0.07	1.20	1.68	0.89	1.44	0.96	2.08	0.67
0.29	0.29	0.19	0.44	0.23	0.24	0.21	0.16	0.00	1.31	2.15	0.69	1.29	0.82	2.14	0.69
0.26	0.21	0.18	0.36	0.19	0.18	0.23	0.24	0.00	1.95	2.44	0.72	1.10	1.00	1.60	0.67
0.29	0.22	0.19	0.36	0.14	0.14	0.14	0.09	0.04	1.32	1.53	0.80	1.57	1.36	2.57	0.48
0.31	0.29	0.25	0.45	0.15	0.14	0.19	0.12	0.00	1.21	1.44	0.69	1.93	1.78	2.37	0.57
0.38	0.25	0.21	0.36	0.22	0.22	0.22	1.18	0.13	1.64	2.00	1.05	1.14	0.95	1.60	0.70
0.37	0.16	0.14	0.34	0.16	0.16	0.16	0.21	0.13	2.31	2.64	1.10	1.00	0.88	2.12	0.57
0.41	0.15	0.12	0.45	0.14	0.14	0.14	0.27	0.09	2.74	3.42	0.91	1.07	0.86	3.21	0.60
0.34	0.22	0.19	0.38	0.15	0.15	0.15	0.26	0.07	2.00	2.42	0.90	1.46	1.26	2.53	0.66
0.39	0.14	0.12	0.34	0.14	0.14	0.14	0.25	0.15	2.80	3.25	1.02	1.00	0.86	2.43	0.56
0.31	0.20	0.16	0.38	0.17	0.17	0.17	0.34	0.04	2.00	2.62	0.81	1.18	0.94	2.23	0.74
0.33	0.18	0.15	0.43	0.14	0.13	0.23	0.36	0.02	2.72	3.40	0.77	1.29	1.15	1.90	0.69
0.39	0.18	0.13	0.48	0.15	0.14	0.21	0.24	0.05	2.17	3.00	0.81	1.20	0.93	2.29	0.56
0.36	0.21	0.17	0.42	0.16	0.15	0.21	0.18	0.07	1.71	2.12	0.86	1.31	1.14	2.00	0.55
0.30	0.24	0.20	0.40	0.25	0.26	0.21	0.29	0.00	2.00	2.65	0.75	0.96	0.77	1.90	0.85
0.42	0.24	0.21	0.37	0.23	0.23	0.23	0.40	0.16	2.50	3.05	1.14	1.04	0.91	1.60	0.92
0.41	0.26	0.17	0.47	0.15	0.15	0.15	0.23	0.08	1.58	2.40	0.87	1.73	1.13	3.14	0.61
0.26	0.27	0.23	0.42	0.23	0.23	0.23	0.25	0.00	1.67	2.17	0.62	1.17	1.00	1.83	0.80
0.36	0.26	0.21	0.44	0.19	0.18	0.22	0.23	0.05	1.65	2.14	0.82	1.37	1.17	2.00	0.69
0.53	0.24	0.15	0.51	0.21	0.21	0.21	0.22	0.17	1.83	2.73	1.04	1.14	0.70	2.43	0.64
0.50	0.26	0.22	0.47	0.16	0.16	0.16	0.17	0.17	1.54	1.73	1.06	1.63	1.38	3.00	0.61
0.46	0.27	0.24	0.47	0.18	0.18	0.18	0.14	0.13	1.44	1.65	0.98	1.50	1.33	2.61	0.63
0.40	0.28	0.21	0.35	0.14	0.15	0.13	0.27	0.15	1.57	2.28	1.14	2.00	1.40	2.70	0.68
0.39	0.27	0.15	0.43	0.15	0.15	0.15	0.28	0.09	1.60	3.06	0.91	1.80	1.00	2.86	0.64
0.36	0.14	0.12	0.29	0.12	0.11	0.19	0.31	0.15	3.00	3.67	1.25	1.17	1.10	1.50	0.59
0.28	0.18	0.13	0.38	0.12	0.11	0.16	0.29	0.00	2.22	3.30	0.73	1.50	1.18	2.37	0.57
	0.31	0.24	0.59	0.16	0.15	0.20			1.35			1.94	1.60	2.95	
0.47	0.36	0.27	0.57	0.15	0.15	0.15	0.12	0.07	1.11	1.37	0.83	2.40	1.80	3.80	0.59
0.62	0.33	0.25	0.50	0.23	0.23	0.23	0.36	0.27	1.90	2.48	1.24	1.44	1.09	2.17	0.93
0.47	0.35	0.27	0.60	0.18	0.19	0.15	0.12	0.05	1.17	1.45	0.78	1.94	1.42	4.00	0.66
0.40	0.27	0.21	0.46	0.21	0.22	0.18	0.20	0.08	1.60	2.10	0.87	1.29	0.96	2.55	0.72
0.32	0.20	0.17	0.26	0.21	0.18	0.27	0.25	0.14	2.00	2.60	1.23	0.95	0.94	0.96	0.67
0.28	0.22	0.23	0.19	0.28	0.25	0.38	0.32	0.15	2.32	2.52	1.47	0.79	0.92	0.50	0.90

As is well known cholesterol is a protective agent against certain hemolytic substances but only when free (21). A knowledge of the relation of free to bound cholesterol seemed therefore important in considering the increased hemolysis in anemia

TABLE II.

Blood Lipoids per 100 Cc., before and after Splenectomy.

Case.	Corpuscles.	Total fatty acids.			Lecithin.			Cholesterol		
		Whole blood.	Plasma.	Corpuscles.	Whole blood	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.
	per cent	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Anemics.										
1. M.										
Before........	22	0.32	0.27	0.38	0.25	0.16	0.57	0.13	0.11	0.21
After..........	26	0.31	0.24	0.40	0.28	0.16	0.62	0.21	0.20	0.23
2. Ba.										
Before........		0.30			0.21			0.18		
After..........	20	0.39	0.38	0.40	0.33	0.22	0.62	0.21	0.20	0.23
3. W.										
Before........		0.24			0.15			0.15		
After..........	31	0.34	0.30	0.43	0.28	0.15	0.59	0.18	0.16	0.20
4. Be.										
Before........	14	0.35	0.35	0.35	0.35	0.16	0.16	0.15	0.15	0.15
After..........	17	0.36	0.33	0.50	0.50	0.18	0.30	0.13	0.13	0.13
Others.										
1. L.										
Before........	37	0.46	0.49	0.41	0.24	0.20	0.31	0.16	0.14	0.19
2 weeks after..	34	0.47	0.44	0.53	0.32	0.22	0.51	0.19	0.19	0.19
2. C.										
Before........	29	0.68	0.55	0.90	0.28	0.16	0.54	0.16	0.14	0.20
After.........	30	0.62	0.54	0.80	0.31	0.22	0.52	0.17	0.15	0.22

especially as in most of the cases examined the values for total cholesterol were abnormally low. Determinations were therefore made of the balance between free and bound cholesterol in a series of the cases examined above. The results of the analyses are given in Table III.

TABLE III.

Cholesterol and Cholesterol Esters per 100 Cc., in the Blood Plasma.

Case.	Disease.	Total.	As ester.	
		mg.	*mg.*	*per cent*
M. A.	Pernicious anemia.	180	112	62
"	" "	140	83	60
"	" "	220	114	52
P. B.	" "	160	100	62
A. D.	" "	140	90	64
"	" "	170	110	65
M. C.	" "	140	100	72
"	" "	150	100	67
F. B.	" "	230	150	65
E. T.	" "	210	120	60
"	" "	160	100	62
A. F.	*Botriocephalus* anemia.	120	77	64
"	" " (after removal).	150	100	67
M. G.	Secondary anemia.	150	88	59
J. F.	Carcinoma.	233	82	35
A. B.	"	181	51	28
Normal average.				59

RESULTS AND DISCUSSION.

Total Fatty Acids.—The total fatty acids are in normal amounts in the majority of the cases studied. Relatively to lecithin and cholesterol, however, they are almost always high, particularly in the plasma, as is brought out by the ratio total fatty acids: lecithin. In Case V the total fatty acids were above the normal value and it is interesting to note that this was the only case in which the Wassermann reaction was positive. In Case IX there were persistently high values in the corpuscles. This patient had an aplastic anemia, grew progressively worse, and died 2 weeks after leaving the hospital. In one case of carcinoma (XVI) the values were high in the plasma.

Lecithin.—The lecithin values in the plasma were either below normal or near the lower normal limit, never high. In the corpuscles the values were essentially normal with occasional high figures.

In the plasma of Cases II, III, and IV, in all of which the percentages of the corpuscles were very low, there were constantly low values. It is notable in these cases that the corpuscle values were normal. There were occasional low values in the other primary anemias and in one of the cases of carcinoma. In the corpuscles the only low values were in two cases of carcinoma, particularly in Case XVI. Case XI (*Botriocephalus* anemia) showed marked increase in lecithin in both plasma and corpuscles following delivery of the worm, the increase keeping pace with the increase in corpuscles. In the fatal case (IX) the lecithin in the plasma increased as the percentage of corpuscles diminished.

Cholesterol.—The values for cholesterol in the plasma were characteristically low in both primary and secondary anemia. The low values were also frequently seen in the corpuscles but do not appear to be characteristic—in fact there was the same tendency as in the case of lecithin for the corpuscle values to remain normal.

Fat.—The fat was almost always high in the plasma and frequently so in the corpuscles. The high values for fat account for the normal level of total fatty acids and total lipoids in cases where the lecithin and cholesterol were low.

Total Fatty Acids: Lecithin.—The values for this ratio were generally high—due rather to low values for lecithin than to high values for total fatty acids.

Lecithin: Cholesterol.—The values for this ratio were generally rather high in pernicious anemia—due to relatively low values for cholesterol. In Case II and in two cases of carcinoma the value was low. In secondary anemia the values were generally normal. For the reason mentioned high values were more frequent in the plasma and occur in secondary as well as primary anemia. It is interesting to note that in the cases of carcinoma the values in the corpuscles were all low.

Total Lipoids (Plasma).—This value was within normal limits in all but Cases V, XII, and XVI. In Case V the high value was due mainly to fat, although the cholesterol value was above the normal average. In Case XII (and also in Case XVI) (lymphatic leukemia—white cells 107,200) the high value was again due to fat.

The Blood Lipoids after Splenectomy.

Total Fatty Acids.—The results are irregular. Out of the six cases examined there was a notable increase in total fatty acids in the whole blood in two cases and a decrease in one (not an anemic). In the corpuscles there was a notable increase in two out of the four on which results were obtained and a decrease in one. The plasma values were mostly unchanged—indicating that whatever changes took place were in the corpuscles alone.

Lecithin.—There were notable increases in the whole blood in all cases, in the corpuscles in all but one (not anemia). The plasma values were unchanged except in this last one which showed a slight increase. The increases in the whole blood were therefore due to increases in lecithin in the corpuscles and not in the plasma.

Cholesterol.—There were increases in all but one in whole blood, due as far as available data show to increases in the plasma —the corpuscle values remaining constant.

Cholesterol and Cholesterol Esters in the Plasma of Anemia.— In pernicious anemia and in the secondary anemias except those from carcinoma the percentage of cholesterol combined as ester, while above the normal average, was not much if any above the normal limits of variation. In the two cases of anemia from carcinoma the values were very low—which appears to be characteristic of carcinoma (22). These results give little ground for the assumption that abnormally large combination of cholesterol as ester is a factor in the anemia.

There appears to be no characteristic difference in the blood lipoids in the different types of anemia.

In those samples where the percentages of corpuscles were not below half their normal value the lipoid values for the blood were generally normal throughout. Two exceptions were Case VI (secondary anemia) with a corpuscle percentage of 30 and Case X (diagnosis doubtful) with a corpuscle percentage of 44. In these the cholesterol values were at the lower limit of normal variation and the fat in the plasma was high. When, however, the corpuscles were below half the normal percentage, low values for cholesterol and lecithin and high values for fat were found.

In the order of the frequency of their occurrence and of their magnitude the abnormalities in the whole series of cases were (1) high values for fat in plasma, (2) low values for cholesterol in plasma and with less frequency in the corpuscles, and (3) low values for lecithin in the plasma. Thus the marked abnormalities were mainly in the plasma, the corpuscles in anemia as in most other conditions examined tending to preserve a constant lipoid composition.

The low values for lecithin occurred in almost all cases along with a high value for fat and a low corpuscle percentage which is regarded as significant in view of the recent findings (14) that the change of fat to lecithin is an early stage in fat metabolism and that this change is brought about by the corpuscles. As the corpuscles decreased in number they were unable to keep this function up to its normal level and there was an accumulation of fat with a corresponding decrease of lecithin. There was frequently a parallelism in individual cases between changes in the number of corpuscles and in the lecithin values in the plasma. Thus in Case I with percentage of corpuscles of 17, 18, and 20, the plasma lecithin values were 0.18, 0.19, and 0.25; in Case II with corpuscle percentages 10 and 9 the lecithin values were 0.14 and 0.12; in Case III corpuscles 16, 9, and 18, lecithin 0.19, 0.12, and 0.16; Case XI (*Botriocephalus*) corpuscles 12, 19, 20, 30, lecithin 0.12, 0.13, 0.24, and 0.27.

Other interpretations are possible for the high fat values.

1. These values together with the low cholesterol values may be a general accompaniment of a lowered vitality. Thus similar high fat values have been reported in the anemia of rabbits produced by bleeding (23) although in this case it might equally well be claimed that the high values were due to the deficiency of corpuscles.

2. The value fat is a residual value and includes not only what is ordinarily termed fat (glycerides of the fatty acids) but also all other forms in which the fatty acids may occur in the blood other than as phosphatides and cholesterol esters, that is as soaps, free fatty acids, or as unknown combinations of these with the other constituents of the plasma as for example with the proteins. The probable presence of other combinations than fat or free fatty acids is given some support by the fact that the plasma

in anemia is generally clear, which could scarcely be the case if this amount of fat were free. There is thus the possibility that an abnormally large amount of a toxic lipoid may be held combined in the plasma. Those cases in which there is a high fat value where the lecithin values are normal and the corpuscle values are above half the normal percentage might then be regarded as earlier stages in the anemia in which, although the toxic lipoid is present in quantity, the destruction of the corpuscles has not proceeded far enough to affect the metabolism of the fat. Of course no claim can be made that all high values for fat are due to the presence of toxic lipoids; for as has already been pointed out (24) high fat values without lipemia are also found in diabetes.

The interpretation of the low cholesterol which was found to be a characteristic feature of the plasma of severe anemia and occurred sometimes in the corpuscles is not altogether clear. Cholesterol acts as an antihemolytic against agents like the saponins, cobralysin, etc., but there is no satisfactory evidence that it protects against hemolysis by the unsaturated fatty acids. In so far, however, as it may act in this capacity the low values may explain the hemolysis. Also the cholesterol is generally lower than the lecithin as shown by the high values for the ratio lecithin: cholesterol which supports the above idea since lecithin and cholesterol have been shown to act antagonistically in certain cases of hemolysis—the excess of lecithin aiding hemolysis. On the other hand low cholesterol values have been claimed (25) to be characteristic of low vitality. The fact that improvement in the clinical condition generally resulted in increased cholesterol values might be taken as supporting either explanation. A possible relation of the spleen to the low cholesterol values is indicated by the fact that with its removal the plasma cholesterol increases. The low value for cholesterol may act indirectly. Mueller (26) has advanced the idea that one function of the blood cholesterol is to combine with toxic fatty acids. It is possible that in the absence of normal amounts of cholesterol—apparently a characteristic of anemia—the toxic fatty acids accumulate in the blood, either as such or more probably in some sort of combination in which, however, they are more or less free to exert their harmful effects. It is not necessary to assume the presence

of an abnormal fatty acid. Oleic acid—the commonest of the fatty acids of the food fat—is as active in this respect as any fatty acid known (2). Normally the animal organism is able to take care of large quantities of olein and to prevent the amounts of free oleic acid or oleic soap, always present in the blood, from reaching toxic proportions, but it has been shown (8) that it is possible in normal animals to produce conditions resembling anemia by continuous feeding of oleic acid or olein, indicating that this mechanism may be made to fail from overwork, and it may be that similar conditions prevail in anemia. Except for the occasional low cholesterol values which do not appear to be characteristic, there is nothing in the lipoid values to indicate that there is any abnormality or special vulnerability in the corpuscles.

Removal of the spleen resulted in increased total fatty acids and lecithin in the corpuscles and of cholesterol in the plasma. The results are approximately the same in anemics and in non-anemics.

SUMMARY.

The blood lipoid values in anemia were found to be normal, or nearly so, as long as the percentage of blood corpuscles remained above half the normal value. When the percentage was below this level abnormalities appeared which, in the order of their magnitude and also of the frequency of their occurrence were (1) high fat in the plasma, (2) low cholesterol in the plasma and occasionally in the corpuscles, and (3) low lecithin in the plasma.

The lipoid composition of the corpuscles was found to be normal in almost all cases. There was therefore nothing in their composition to indicate abnormal susceptibility to hemolysis.

Removal of the spleen resulted in increased total fatty acids and lecithin in the corpuscles and of cholesterol in the plasma. The results were essentially the same whether the patients had anemia or not.

The relation between free and bound cholesterol was found to be within normal limits in all cases of anemia except the two cases in which there was carcinoma, thus giving little support to the assumption that an abnormally great combination of cholesterol as ester is a factor in the production of anemia.

The low values for lecithin and the high values for fat which were generally most marked in these cases where the blood corpuscle percentages were lowest are regarded as due to deficient fat assimilation in the blood resulting from the lack of sufficient corpuscles to bring about the change of fat to lecithin which has been found to be one function of the corpuscles.

While the results offer no certain evidence that abnormalities in the blood lipoids are responsible for anemia, the low values for cholesterol, which is an antihemolytic substance, and the high fat fraction, which may indicate the presence of abnormal amounts of hemolytic lipoids in the blood, are possible causative factors of which further investigation is desirable.

The more important data regarding the individual cases are given below. For convenience a summary of the notable lipoid findings are included with the data on each case.

Case Histories.

Case I.—M. A., No. 3718 (pernicious anemia), age 36. For 4 years had had recurrent attacks of vomiting and abdominal pain associated with pallor and weakness with slight improvement between attacks. There was no loss of weight. Very recently she has had numbness of the extremities. Marked anemia. Liver palpable, spleen palpable. Hemoglobin 35 per cent, erythrocytes 1,300,000, white count 4,900, small mononuclears 50 per cent, anisocytosis and poikilocytosis. Wassermann reaction negative. Temperature 99–100°, occasionally 101° till the week before discharge. Improved clinically, discharged after 7 weeks. Hemoglobin 59 per cent, erythrocytes 2,202,000, white count 3,800, small mononuclears 22 per cent, 2 normoblasts, 1 megaloblast. Patient well at present time.

Lipoids.—The most marked abnormality in the blood lipoids of this patient was a low cholesterol value in the plasma, and the essential difference between the blood lipoids when ill and when finally discharged improved was an increase of cholesterol in the plasma.

Case II.—P. B., No. 4223 (pernicious anemia), age 46. In 2 years previous had had three characteristic attacks of pernicious anemia. Was subject to severe cardiac pain in anemic periods. No loss of weight. Spleen very large. Liver palpable. Wassermann negative. Metabolism 21 per cent above normal. He had seven transfusions in a period of 4 months, was discharged with hemoglobin 51 per cent, erythrocytes 2,344,000. He died at home 5 months later.

Lipoids.—Very low lecithin in plasma and low cholesterol both in plasma and corpuscles, high fat in plasma. Total lipoids normal.

Case III.—A. D., No. 4064 (pernicious anemia), age 63. Second attack of weakness, shortness of breath, indigestion, and typical picture of pernicious anemia (was a patient at this hospital during the first attack). At present hemoglobin 42 per cent, erythrocytes 1,400,000, white count 3,300. Wassermann negative. Spleen palpable. Metabolism 12 per cent above normal. He was given several injections of diarsenide and transfused three times with citrated blood. He improved slowly. On discharge hemoglobin 68 per cent, erythrocytes 2,140,000, white count 6,000. Temperature on days specimens were obtained 99° to normal. Patient well at present.

Lipoids.—Lecithin low in plasma, cholesterol low throughout, fat high in plasma.

Case IV.—M. C., No. 4181 (pernicious anemia), age 51. For 7 months had noticed weakness, pallor, dyspnea, and palpitation. For 3 to 4 months attacks of nausea and vomiting. Well developed and nourished, marked yellowish pigmentation to skin. Tongue smooth and glossy. Liver and spleen palpable. Wassermann negative. Hemoglobin 33 per cent, erythrocytes 1,530,000, white count 3,100. Metabolism 6 per cent below normal. Patient improved following two transfusions and two injections of diarsenide. Discharged after 9 weeks. Hemoglobin 82 per cent, erythrocytes 3,500,000.

Lipoids.—Lecithin low in plasma, cholesterol low in plasma, corpuscles normal, total fatty acids and fat high in plasma. With treatment the total fatty acids and fat in the plasma diminished, while lecithin and cholesterol increased. The value of the ratio total fatty acids: lecithin diminished while that of lecithin: cholesterol remained constant.

Case V.—F. B., No. 4063 (pernicious anemia, syphilis), age 63. Increasing weakness for 4 months with palpitation and dyspnea, occasional paresthesia of feet. Marked brown pigmentation of skin. Smooth glossy tongue. Spleen easily palpable. Wassermann reaction positive. Hemoglobin 48 per cent, erythrocytes 1,400,000, no blasts. Improved after three injections of diarsenide. Discharged after 7 weeks. Hemoglobin 92 per cent, erythrocytes 3,500,000.

Lipoids.—The only notable feature of the blood lipoids in this case was high fat in the plasma.

Case VI.—M. G., No. 4043 (acute bronchitis, acute fibrinous pleurisy, secondary anemia), age 55. Wassermann negative. Hemoglobin 40, erythrocytes 4,728,000, white count 24,400. Improved under treatment.

Lipoids.—Low cholesterol throughout, otherwise the lipoids were normal.

Case VII.—C. S., No. 4059 (telangiectasis of face, nasal and buccal membranes, multiple and with recurring hemorrhages, secondary anemia), age 53. Severe bleeding from nose, and tarry stools. Spleen not palpable. Wassermann negative. Hemoglobin 55 per cent, erythrocytes 3,368,000, white count 9,300. Improved under treatment.

Lipoids.—High fat in plasma. High total lipoid.

Case VIII.—L. H., No. 4061 (ulcer of stomach, secondary anemia), age 29. Several hemorrhages during previous 2 weeks. Tarry stools. Spleen not palpable. Wassermann negative. Hemoglobin 45 per cent, erythrocytes 2,696,000, white count 7,000.

Lipoids.—Slightly low cholesterol in plasma.

Case IX.—E. T., No. 3768 (pernicious anemia, hemorrhagic stomatitis), age 30. Frequent attacks of jaundice since childhood. 10 days prior to entrance her gums commenced to bleed. Followed 2 days later by epistaxis, chills, and fever. Mouth ulcerated. Breath foul. Few ecchymotic áreas on lower limbs. Marked tenderness over both tibias. Spleen not palpable. Wassermann negative. Hemoglobin 60 per cent, erythrocytes 2,900,000, white count 7,500, small mononuclears 89 per cent, guaiac negative in stools. No hemorrhage into retina. Patient grew progressively worse. Discharged to relatives. Died at home 2 weeks later following severe bleeding from mouth. On discharge hemoglobin 25 per cent, erythrocytes 1,248,000, white count 4,650, small mononuclears 66 per cent, 3 eosinophil myelocytes.

Lipoids.—Cholesterol low in plasma and corpuscles. Fat high in corpuscles.

Case X.—T. H., No. 3729 (anemia—pernicious (?) secondary to gasoline fumes?), age 17. For 1 year has worked in a garage, inhaling a great deal of smoke from cars, working with gasoline. Pale for 8 weeks. 6 weeks prior to present began to feel weak, lost his appetite, and would often be nauseated and vomit. 3 weeks before entrance he gave up his work because of increasing weakness, dyspnea on exertion, and dizziness accompanied by headache. Moderate loss of weight. Spleen palpable. Wassermann reaction negative. Hemoglobin 36 per cent, erythrocytes 1,300,000, white count 6,400, color index 1.4. Discharged after 2 months. Hemoglobin 106 per cent, erythrocytes 4,600,000. Reported 9 months later. Hemoglobin 119 per cent, erythrocytes 6,136,000.

Lipoids.—Cholesterol low throughout. Lecithin low in plasma. Fat abnormally high.

Case XI.—A. F., No. 3709 (anemia secondary to *Botriocephalus latus*), age 33. Admitted during third attack of vomiting, weakness, and increasing pallor. Previous attacks 8 and 2 years ago. In good health in intervals. Marked pallor. Liver and spleen not palpable. Wassermann negative. Hemoglobin 40 per cent, erythrocytes 1,400,000, white count 4,400, small mononuclears 59 per cent, eosinophils 2 per cent. Stool showed ova of *Botriocephalus*. Resistance of red blood corpuscles, minimal 0.51, maximal 0.33. 1 week later delivered of the heads of four worms and 40 meters of worm. Discharged after 5 weeks. Hemoglobin 88 per cent, erythrocytes 4,000,000, white count 6,000, 2 normoblasts.

Lipoids.—At first lecithin and cholesterol low especially in plasma, with high fat in plasma. After removal of the worm, the blood lipoids became normal except the cholesterol which remained somewhat low.

Case XII.—E. S., No. 4180 (lymphatic leukemia), age 44. Not well for 2 years, general glandular enlargement. Spleen enlarged to umbilicus. Uterine fibroids. Liver large. Wassermann reaction negative. Hemoglobin 75 per cent, erythrocytes 2,510,000, white count 107,200. Small mononuclears 96.5 per cent. One megaloblast. Referred for radium treatment.

Lipoids.—The striking feature in the blood lipoids is the high fat with the resulting high total lipoids. The lecithin and cholesterol are normal.

Case XIII.—M. L. D., No. 3761 (gastric ulcer, secondary anemia), age 34. Recurrent gastric distress. 4 days prior to entrance vomited about one quart of blood and passed tarry stool. Second hematemesis on day before admission. Marked pallor and emaciation. Liver and spleen not palpable. Wassermann reaction negative. Hemoglobin 73 per cent, erythrocytes 3,700,000. Improved under medical treatment.

Lipoids.—Low cholesterol and high lecithin throughout.

Case XIV.—A. T., No. 3982 (internal hemorrhoids, secondary anemia), age 24. Bleeding from rectum for 6 years with severe secondary anemia. Liver and spleen not palpable. Hemoglobin 36 per cent, erythrocytes 2,500,000, white count 15,600. Transferred for operation.

Lipoids.—Normal.

Case XV.—A. B., No. 3565 (carcinoma of stomach, hernia of abdominal wall, hematuria), age 52. Loss of weight. Rounded mass in epigastrium. Appears anemic but hemoglobin 84 per cent. Bismuth studies show large annular carcinoma of the antrum, entire lesser curvature, and fundus with pyloric obstruction. Wassermann reaction negative.

Lipoids.—Lecithin low in corpuscles, fat high in plasma and corpuscles.

Case XVI.—J. F., No. 3368 (carcinoma of stomach). For 8 months loss of weight and vomiting. Large mass in epigastrium. Guaiac reaction in stool positive. Bismuth study shows infiltration in cardiac end of stomach.

Lipoids.—High fat and cholesterol in plasma and corpuscles. Low lecithin in corpuscles. High total lipoids.

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STUDIES ON ENZYME ACTION.

XIV. FURTHER EXPERIMENTS ON LIPOLYTIC ACTIONS.

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CONTENTS.

Inactivation Experiments.

In the further study[1] of the ester-hydrolyzing enzymes or lipases, a systematic investigation of the factors which control the loss or destruction of this activity was undertaken.[2]

The preparations used were obtained from castor beans and consisted of:

1. An albumin-like body more active toward ethyl butyrate than toward glyceryl triacetate under certain definite conditions, used in the form of a clear dialyzed and filtered water extract of oil- and husk-free castor beans, 0.5 gm. to 60 cc. denoted as *esterase* preparation.

2. A globulin-like body, more active toward glyceryl triacetate than toward ethyl butyrate under conditions similar to those

[1] The previous work on this subject was published in a series of papers in *J. Am. Chem. Soc.*, 1912–15; a partial summary was given in *Proc. Nat. Acad. Sc.*, 1915, i, 136.

[2] A preliminary paper was published in *Proc. Nat. Acad. Sc.*, 1916 ii, 557.

under which the esterase was studied, prepared by extracting the water-extracted castor beans with 1.5 normal sodium chloride solution, 1.0 gm. to 100 cc., and dialyzing until salt-free. The preparation consisted of a suspension of the material in water and is denoted here as *lipase* preparation.

The activity tests were carried out with 1.0 cc. of ethyl butyrate or 0.5 cc. of glyceryl triacetate at 38° for periods of time between 18 and 48 hours, and the results given as the number of cc. of 0.1 N alkali required to neutralize the acid produced, with phenolphthalein as indicator, all suitable corrections for blanks having been introduced; or in other words, the amount of acid in tenths of millimols formed from the ester by the action of the enzyme.

The hydrogen ion concentrations were determined by color comparison with standard solutions using suitable indicators.[3] For the purposes in view it was not considered necessary to use the potentiometer also for these measurements.

(*a*) *Inactivation by Acids.*—120 cc. portions of esterase preparation (solution) were treated with 0.1137 N HCl solution.

HCl solution, *cc*.....	0	1.00	2.00	3.00
H^+ concentration...	$10^{-7.0}$	$10^{-4.5}$	$10^{-3.5}$	$10^{-3.0}$
Appearance.........	Clear.	Coagulated.	Turbid.	Slightly turbid.

After standing in the ice box 25 hours they were neutralized to $C_H = 10^{-7.0}$.

0.1 N HCl[4] in excess of calculated quantities, *cc*..	0	0.06	0.13	0.18
Appearance after neutralization..................	Clear.	Very turbid.	Less turbid.	Slightly turbid.
Actions, 35 cc. portions, 43 hrs., ethyl butyrate...	1.93	1.19	0.12	0.10

140 cc. portions of esterase preparation (solution) were treated with 0.1137 N HCl solution.

HCl solution, *cc*........................	0	0.50	1.00
H^+ concentrations........................	$10^{-7.0}$	$10^{-6.0}$	$10^{-5.0}$

[3] Noyes, A. A., *J. Am. Chem. Soc.*, 1910, xxxii, 822. Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1.

[4] These measure the amounts of neutralization due to the alkaline material formed in the inactivations.

After standing in the ice box 20 hours they were neutralized to $C_H = 10^{-7.0}$.

Appearance after neutralization........	Clear.	Slightly turbid.	Very turbid.
Actions, 40 cc. portions, 24 hours, ethyl butyrate..............................	1.69	1.44	1.23

These experiments showed that at $C_H = 10^{-3.5}$ practically all the activity of the esterase preparation was lost in 24 hours, while at $C_H = 10^{-4.5}$ one-third of the activity was lost.

150 cc. portions of lipase preparation (colloidal mixture) were treated with 0.1137 N HCl solution.

HCl solution, *cc*........................	0	5.0	10.0	20.0

After standing in the ice box 19 hours they were brought to $C_H = 10^{-7.0}$ with the calculated equivalent quantity of alkali.

Appearance after neutralization..........	Suspension.	Coagulated.	Coagulated.	Coagulated.
Actions, 50 cc. portions, 24 hours, glyceryl triacetate.....	0.87	0.44	0.36	0.27

160 cc. of lipase preparation were treated with 4 cc. of 1.0 N HCl solution and allowed to stand in the ice box 24 hours. The solid matter went into solution partly, but the mixture was still very turbid. On being neutralized to $C_H = 10^{-7.0}$ a coagulated precipitate was formed. Action, 50 cc. portion, 23 hours, glyceryl triacetate, 0.15; the same mixture without acid treatment, 0.75.

The rate of inactivation of the lipase preparation by acids does not appear to be as great as that of the esterase preparation. With the former the hydrogen ion concentration to which the mixtures were brought ranged from 10^{-2} to 10^{-3} in the different experiments. The physical state of the substance undoubtedly plays a part in determining the rate of inactivation, but it appears to be unquestionable that practically complete inactivation would result with both preparations even in extremely dilute acid solutions in somewhat longer periods of time than those here used.

(*b*) *Inactivation by Bases.*—120 cc. portions of esterase preparation were treated with 0.1037 N NaOH.

NaOH, *cc*....................	0	1.0	2.0	4.0	6.0
H^+ concentration...........	$10^{-7.0}$	$<10^{-11}$	$<10^{-11}$	$<10^{-11}$	$<10^{-11}$

After standing in ice box 26 hours they were neutralized.

Appearance	Clear.	Slightly cloudy.			
Actions, 40 cc., 20 hours, ethyl butyrate	1.33	0.65	0.19	0.04	0.04

120 cc. portions of esterase preparations were treated with 0.1037 N NaOH.

NaOH, *cc.*	0	0.12	0.24	0.48	1.00
H^+ concentrations	$10^{-7.0}$	$10^{-7.5}$	$10^{-8.5}$	$10^{-10.5}$	10^{-11}
After standing in the ice box 67 hours, H^+ concentrations	$10^{-7.0}$	$10^{-7.2}$	$10^{-7.5}$	$10^{-8.2}$	10^{-11}
Brought to neutrality, alkaline excess in cc. of 0.1 N NaOH	0	0.06	0.06	0.32	0.22
Actions, 30 to 35 cc. 23 hours, ethyl butyrate	1.10	1.09	0.92	0.21	0.16

A 140 cc. portion of esterase preparation was brought to $C_H = 10^{-8.0}$, allowed to stand in the ice box 20 hours (H^+ concentration remaining practically unchanged), neutralized (0.26 cc. of 0.1 N NaOH in excess present compared to calculated amount), and the actions tested; 40 cc., 24 hours, ethyl butyrate, 1.45; untreated preparation 1.69.

These experiments show that keeping the esterase preparations at $H^+ = 10^{-8}$ for the periods indicated caused only slight inactivation compared with the neutral solution, but the more alkaline solutions became inactivated more rapidly, all but 10 per cent being lost at $C_H = 10^{-11}$, and even this being destroyed in the still more alkaline solution. The solid matter in the lipase preparation mixtures dissolved to a great extent at $C_H = 10^{-11}$, only a slight turbidity remaining. A heavy white precipitate was formed again on neutralizing these solutions.

Portions of lipase preparation mixtures were treated as indicated and the actions tested; 47 hours, 25 cc., glyceryl triacetate.

Actions.

1. No treatment, stood 18 hours at room temperature.......1.02
2. Brought to $C_H = 10^{-11}$, neutralized at once, stood 18 hours before testing.......0.52
3. Brought to $C_H = 10^{-11}$, stood 18 hours, then neutralized and tested.......0.48

150 cc. portions of lipase preparations were treated with 0.1037 N NaOH.

NaOH, *cc*	0	5.0	10.0	20.0
Appearance	Unchanged.	Slightly turbid.		
After standing in the ice box for 23 hours they were neutralized and tested; 50 to 60 cc., equivalent amounts. 22 hours, glyceryl triacetate	0.76	0.50	0.32	0.29

These experiments show that greater amounts of alkali were needed to produce the corresponding inactivation with the lipase preparation than with the esterase preparation. They also show that the effect, 50 per cent loss in activity, caused by the $H^+ = 10^{-11}$ is produced at once, standing 18 hours at room temperature causing no further inactivation.

In the treatment of the esterase preparation with dilute acid or alkali there was a marked buffer action apparent, tending to bring the solutions to the neutral point. Alkali and acid were produced in the solutions so that when the mixtures were neutralized again, smaller quantities of the alkali and acid were required than were calculated from the quantities of acid and alkali originally added. These changes were not observed with the lipase preparations.

In some of these experiments the mixtures without and with treatment with acids and alkalis were titrated by the formol method. No increase in the formol titration was observed after such treatments, so that the production of amino-carboxyl groupings by the breaking up of peptide linkings is negligible with the concentrations of acid and alkali used.

(*c*) *Inactivation by Neutral Salts.*—An extended study of the effect produced by a number of different salts at various concentrations on the activity of the castor bean preparations, mainly toward ethyl butyrate, showed that some of the salts retarded the actions very markedly, others to a less extent, while some accelerated the action of the enzyme.[5] While the studies were incomplete in that the determination of the hydrogen ion concentrations was not included and only a limited number of experiments were made with esters other than ethyl butyrate, still regularities with

[5] Falk, K. G., *J. Am. Chem. Soc.*, 1913, xxxv, 601. Falk, K. G., and Sugiura, K., *ibid.*, 1915, xxxvii, 217.

different series of cations and anions could be observed, but there was no general underlying principle apparent governing the two actions, acceleration and retardation. These seemingly opposite actions may well be different phases of the same phenomenon.

(*d*) *Inactivation by Alcohols and by Acetone.*[6]—Dilute solutions of methyl and ethyl alcohols and of acetone retarded the actions of both preparations, the amount of retardation increasing with the concentration of the alcohol or acetone. Solid preparations made by precipitation and washing with alcohol were always inactive. Solid esterase preparations precipitated and washed with acetone were active in a number of cases, but the activity was very much smaller than that of the corresponding solutions from which they were prepared. Similar solid lipase preparations were always inactive. The addition of glycerol, however, up to a concentration of 25 per cent had no effect upon the activity of the original castor bean preparation.

(*e*) *Inactivation by Esters*[7].—The actions of the esters paralleled those of the alcohols. Methyl acetate inhibited the action more than ethyl butyrate, while glyceryl triacetate had very little, if any, such action. It was pointed out previously that these actions serve to explain part of the selective actions of the lipases which have been described in the past and that the action of the substrate on the enzyme must in all cases be taken into account when considering the properties of enzymes.

(*f*) *Inactivation by Heat.*—The original castor bean preparation containing the enzymes was inactivated by heating with water for a few minutes at 100°. The same preparation heated dry at 100–110° lost 50 to 80 per cent of its activity. The same loss in weight brought about in a vacuum desiccator over phosphorus pentoxide was not accompanied by loss of activity. Drying first and then heating (the latter causing only 0.1 to 0.2 per cent increased loss in weight) caused 50 to 80 per cent loss in activity.[8]

The esterase preparation, $C_H = 10^{-7.0}$, on being boiled vigorously for 5 minutes and then cooled, showed $C_H = 10^{-8.5}$, and a somewhat cloudy appearance. After being neutralized, no action

[6] Falk, *J. Am. Chem. Soc.*, 1913, xxxv, 616 and 1904. Falk and Sugiura, *ibid.*, 1915, xxxvii, 217.

[7] Falk, *J. Am. Chem. Soc.*, 1913, xxxv, 616.

[8] Falk and Sugiura, *J. Am. Chem. Soc.*, 1915, xxxvii, 217.

was observed toward ethyl butyrate. The lipase preparation when boiled showed no change in hydrogen ion concentration while the activity was lost. It was shown previously that the esterase preparation on standing lost its activity very much more rapidly than did the lipase preparation, which, perhaps because of its physical condition, retained its activity very nearly unchanged for several days.

Theoretical Considerations.

The different ways in which the esterase and lipase preparations may be inactivated make it appear at first sight as if different reactions occurred in the inactivations. If, however, a definite chemical group is responsible for a definite enzyme action, it might perhaps be more reasonable to assume that inactivation followed a definite reaction. The preparations were essentially protein in character. There is no evidence that a dehydration, or loss of the elements of water, caused inactivation. Some of the reactions indicated that a possible hydrolysis may be a cause of inactivation. With proteins, hydrolysis is generally taken to occur with the $-CO-NH-$ group which goes over into the $-COOH\ NH_2-$groups. Experiments with all the inactivations in no case showed an increase in the formol titration such as would be expected in this reaction, and therefore makes the assumption of such a hydrolysis improbable. Coagulation of the material accompanied some of the inactivations. The physical change alone does not appear satisfactory as an explanation, some change in chemical structure unquestionably accompanying or producing the physical phenomenon. Furthermore, the lipase material in suspension in water showed the same activity as when dissolved in 1.5 normal sodium chloride solution when tested immediately.

The explanations of the chemical changes accompanying inactivation so far suggested are not satisfactory. The reagents used are simple. It is difficult to conceive of a very deep-seated chemical reaction taking place under so many different conditions, none of a complex nature. The only chemical change which appears probable under these conditions is that involving a simple rearrangement within the molecule, such as a tautomeric change involving the change in position of the hydrogen atom.

In considering the structure of proteins it is evident that such a rearrangement is possible in the peptide linking.

The hypothesis to be suggested is that the active grouping of the esterase and lipase preparations is of the enol-lactim structure, $-C(OH)=N-$, the specificities being dependent in part upon the groups combined with the carbon and nitrogen, and that inactivation consists primarily in a rearrangement to the keto-lactam group $-CO-NH-$.

The further work to be described here will show in brief that in the presence of simple peptides, esters are hydrolyzed under conditions which favor the production in the former of the enol-lactim grouping; that a substance, ethyl imidobenzoate, having the enol-lactim structure, possesses marked ester-hydrolyzing action as well as certain properties strikingly analogous to those of the naturally occurring lipolytic enzymes; and finally, that under conditions under which the occurrence or formation of the enol-lactim structure might be expected (action of alkali) ester-hydrolyzing substances are produced from proteins.

Action of Dipeptides and of Amino-Acids.

In previous papers of this series[9] the ester-hydrolyzing actions of a number of amino-acids and peptides were studied. The work dealt more particularly with the specific actions of these substances on a number of esters, and their behavior as a whole was studied without attempting to analyze more closely the groups responsible for the actions. In this paper the actions of some dipeptides will be described from the point of view of their structural configuration, and in this connection a similar study of the amino-acids was necessary.

According to the hypothesis suggested in the last section, ester-hydrolyzing action may be caused by the enol-lactim grouping $-C-(OH)=N-$ which loses this property in rearranging to the tautomeric keto-lactam grouping $-CO-NH-$. The action of alkali is generally considered to produce the former grouping in such organic compounds, perhaps with the simultaneous replacement of the hydroxyl hydrogen by metal. The best studied

[9] Falk, K. G., and Nelson, J. M., *J. Am. Chem. Soc.*, 1912, xxxiv, 828. Hamlin, M. L., *ibid.*, 1913, xxxv, 624 and 1897.

example of this appears to be isatin, but there is considerable evidence, much of it indirect it is true, that with compounds containing such groups, an equilibrium between the two tautomeric forms exists, the enol-lactim form predominating in alkaline solution, the keto-lactam form in neutral or acid solution. The hydrolytic actions of some of the simpler dipeptides chiefly on ethyl butyrate and glyceryl triacetate, since these were the esters used in the study of the naturally occurring lipases, as well as on a few other esters in neutral and slightly alkaline solutions, were measured. The results found are given in Table I. The titrations were carried out by the formol method, and the results all corrected for blanks done under the same conditions. The hydrogen ion concentrations given in the headings refer to the solutions as made up at the beginning of the experiments by the addition of hydrochloric acid or sodium hydroxide; in a number of experiments the hydrogen ion concentrations were determined also at the end of the reactions and these are indicated by the numbers in parentheses in the body of the table.[10]

The results are given in terms of the amount of acid in tenths of millimols formed at 38° in the lengths of time indicated from 1.0 cc. of ethyl butyrate, 0.5 cc. of glyceryl triacetate, etc.

Certain points appear clearly in the results as given. The actions are very much more marked in the alkaline solutions than in the neutral. At the concentration $C_H = 10^{-9.0}$, 0.00001 normal hydroxyl ions, considerable action was obtained, especially toward the acetates. There is in these cases a decrease in the hydroxyl ion concentration in the course of the experiments, approaching neutrality or going beyond in some cases. It is difficult to judge how far this influences the results, as it unquestionably does. The objection may be raised that the alkalinity of the solutions alone caused the hydrolysis, and that the peptides act only as buffer mixtures to keep the hydroxyl ion concentration predominant as compared to the water ester blanks, which, with the acetates, became neutral or slightly acid in reaction more rapidly. This objection is disposed of by comparison with the results obtained with the amino-acid solutions later

[10] The hydrogen ion concentrations in all the tables are indicated by the Sörensen symbol, pH, for the negative exponent of ten.

TABLE I.

Hydrolytic Actions of Dipeptides on Esters.

Substance.	Volume.	Ester.	Time of action.	Action observed. pH. 6.0	7.0	8.0	9.0
gm.	*cc.*	*cc.*	*hrs.*				
Glycylglycine.		Glyceryl triacetate.					
0.10	25	0.5	26		0.19(5.5)	0.45(6.0)	3.48(7.2)
0.10	25	0.5	19				3.56(8.0)
0.05	25	0.5	19		0.30(5.8)		1.72(7.5)
0.025	25	0.5	19				0.95(6.0)
0.012	25	0.5	19				0.48(5.5)
0.025	25	0.5	6				0.61(8.0)
0.025	25	0.5	24				0.82(6.0)
0.025	25	0.5	48				0.87(5.5)
0.10	25	0.05	22				0.22(7.0)
0.10	25	0.1	22				0.32(6.5)
0.10	25	0.2	22				0.34(6.0)
0.10	25	0.5	22				0.40(5.5)
0.10	25	1.0	22				0.43(5.3)
		Ethyl butyrate.					
0.12	40	1.0	44	0	0.13	0.51	0.84
0.10	25	1.0	26		0(6.0)	0.13(6.5)	0.45(7.8)
0.10	25	1.0	19				0.30(8.0)
0.05	25	1.0	19				0.30(8.0)

0.025	25	1.0	19				0.23(8.0)
0.012	25	1.0	19				0.19(8.0)
0.05	25	0.5 Methyl acetate.	22	0.03(5.0)	0.20(6.0)	1.09(7.5)	1.41(7.5)
0.05	25	0.5 Phenyl "	22	0.09(5.0)	0.46(7.0)	1.02(7.0)	1.44(7.0)
0.055	25	0.5 Methyl "	22				1.54(7.8)
0.055	25	0.5 Ethyl "	22				0.96(8.0)
0.055	25	0.5 Phenyl "	22				1.52(6.5)
0.055	25	0.5 Glyceryl triacetate.	22				2.23(7.0)
0.055	25	0.5 Methyl benzoate.	22				0.15(8.0)
0.055	25	0.5 Ethyl "	22				0 (8.5)
0.055	25	0.5 Ethyl butyrate.	22				0.22(8.0)
0.055	25	0.5 (Gm.) phenyl benzoate.	22				0 (8.0)
0.055	25	1.0 Olive oil.	22				0.23(8.5)
0.055	25	1.0 Cottonseed oil.	22				0 (8.5)
Glycylglycine 0.10	25	1.0 Ethyl butyrate.	19				0.30(8.0)
Alanylglycine 0.10	25	1.0 " "	19				0.36(8.0)
Leucylglycine 0.10	25	1.0 " "	19				0.24(8.0)
Glycylleucine 0.10	25	1.0 " "	19				0.48(8.0)
Leucylleucine 0.10	25	1.0 " "	19				0.34(8.0)
Glycylglycine 0.10	25	0.5 Glyceryl triacetate.	19				3.56(8.0)
Alanylglycine 0.10	25	0.5 " "	19				3.30(8.0)
Leucylglycine 0.10	25	0.5 " "	19				2.45(8.0)
Glycylleucine 0.10	25	0.5 " "	19				2.45(8.0)
Leucylleucine 0.10	25	0.5 " "	19				1.73(7.0)

where the buffer action is presumably the same, but entirely different actions were obtained, both absolutely and relatively. A comparative study of the hydrolytic actions of the dipeptides in themselves also answers the objection.

It is interesting to note that the action toward glyceryl triacetate of glycylglycine at $C_H = 10^{-9.0}$ appears to be proportional to the amount of glycylglycine; but if increasing quantities of ester are used with a definite but small amount of glycylglycine, there is only a small increase in action.[11] Toward ethyl butyrate the action is too small to allow definite comparisons.

The actions of equimolar quantities of the five dipeptides may be compared. The following table gives the actions $\times 10^{-2}$ on ethyl butyrate (1.0 cc.) and glyceryl triacetate (0.5 cc.) of 1 gm. molecule of each of the peptides under the conditions indicated in Table I, as calculated from these results.

	Ethyl butyrate.	Glyceryl triacetate.	Ratio, glyceryl triacetate: ethyl butyrate.
Glycylglycine	4.0	47.0	11.8
Leucylglycine	5.3	48.2	9.1
Glycylleucine	4.5	46.1	10.2
Leucylleucine	9.0	46.1	5.1
Alanylglycine	8.3	44.7	5.4

These results are strictly comparable, except for the differences (molar) in concentration of the peptides in solution, since the hydrogen ion concentrations of all changed practically to the same extent in the course of the experiments. Owing to the small actions actually obtained with ethyl butyrate, the experimental error is relatively much greater for the results with it than for the results with glyceryl triacetate. The constancy of the actions toward glyceryl triacetate is striking, the mean value being 46.4. Expressed in slightly different terms, this means that if the relations hold for all concentrations, 1 gm. molecule of the peptide will hydrolyze 0.464 gm. equivalents of glyceryl triacetate in 19 hours. Toward ethyl butyrate the variation in the actions is greater, the mean being 6.2, or very much less than the action

[11] This is similar to the action of the lipase preparation described by Falk and Sugiura, *J. Am. Chem. Soc.*, 1915, xxxvii, 226.

toward glyceryl triacetate under the given conditions. This is also brought out by the ratio of the action toward the two esters shown in the last column, these varying from 5 to 12, mean 8.3.

The comparative action of the glycylglycine toward different esters may also by summarized. The following list contains the actions $\times 10^{-2}$ of 0.055 gm. of glycylglycine at $C_H = 10^{-9.0}$ on one equivalent of each ester under the conditions as stated.

Phenyl acetate.	3.98	Ethyl acetate.	1.84	Ethyl benzoate.	0
Glyceryl triacetate.	2.84	" butyrate.	0.58	Phenyl "	0
Methyl acetate.	2.40	Methyl benzoate.	0.20		

The general formula for the peptides may be written as follows:

$$\begin{matrix} CHR-CO-NH-CHR' \\ | \qquad\qquad\qquad\quad | \\ NH_2 \qquad\qquad\quad CO_2H \end{matrix} \quad \text{or} \quad \begin{matrix} CHR-C(OH)=N-CHR' \\ | \qquad\qquad\qquad\qquad | \\ NH_2 \qquad\qquad\qquad CO_2H \end{matrix}$$

The groups which may be considered involved in the actions are the amino and carboxyl groups or the central $-CO-NH-$ group or its tautomer. In attempting to separate the actions of these groups, two lines of experimentation were followed. In the first place the actions of the amino and carboxyl groups were masked by studying the behavior of the glycylglycine ester hydrochloride and hydrobromide, and secondly, the actions of the amino and carboxyl groups alone were followed by studying amino-acids, all under the same conditions as those under which the peptides were studied. While these methods permit the studying of the groups alone it must be remembered that they leave out of account the important factor of the influence on the tautomeric equilibrium of the molecule as a whole.

The results on p. 110 were obtained with the glycylglycine ester hydrogen halides.

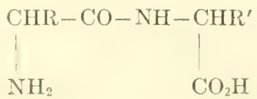
There was only very slight decomposition of the peptide ester as shown by the blanks on immediate titration and after standing. On the other hand, there was a marked tendency for the solution to become neutral on standing. There was not sufficient decomposition of the peptide ester blanks to account for this, the hydrogen halide evidently being involved in the reaction. However, this was apparently slow enough to permit of marked actions being observed with $C_H = 10^{-8.0}$ and $10^{-9.0}$ initially.

pH	6.0	7.0	8.0	9.0
Glycylglycine ethyl ester hydrobromide, ethyl butyrate	0 (6.0)	0 (6.0)	0.64(6.5)	0.10(7.8)
Glycylglycine ethyl ester hydrochloride, ethyl butyrate	0.05(6.0)	0 (5.8)	0 (6.0)	0.02(6.5)
Glycylglycine ethyl ester hydrobromide, glyceryl triacetate	0 (5.2)	0.12(5.8)	0.54(6.2)	0.98(6.2)
Glycylglycine ethyl ester hydrochloride, glyceryl triacetate	0 (5.0)	0.03(5.5)	0.39(5.5)	0.89(6.0)

The lack of action toward ethyl butyrate is significant. The action $\times 10^{-2}$ of 1 gm. molecule of the peptide ester hydrogen halide toward glyceryl triacetate (0.5 cc.) is found to be 19.7 for the hydrobromide and 14.6 for the hydrochloride as compared with the action of 46.4 for the pure peptides. This difference may be accounted for by the more rapid increase in acidity tending to cause a shift of the tautomeric equilibrium to the presumably inactive keto-lactam form, and possibly by the difference in the composition of the molecule.

The second method of studying the influence of the different groups separately is to compare the actions of some amino-acids with the peptides under similar conditions. This may be illustrated by comparing the following formulas for amino-acids and peptides.

A.



B.

By comparing the actions of equivalent amounts of substances of Formulas A and B under comparable conditions, it should be possible to find the action due to the grouping $-CO-NH-$ in A with the possible reservation that this group and the amino and carboxyl groups may exert reciprocal influences upon each other although no direct evidence of such influence has been obtained.

Table II gives the results obtained with some amino-acids.

Since considerable work was published in the previous papers of this series on the hydrolytic actions of amino-acids and their specificity toward certain esters, only a brief discussion of the

TABLE II.

Hydrolytic Actions of Amino-Acids on Esters.

Substance.	Volume.	Ester.	Time of action.	Actions observed. pH. 5.0	6.0	7.0	8.0	9.0
gm.	*cc.*	*cc.*	*hrs*					
Glycine.								
0.033	25	1.0 Ethyl butyrate.	26					0.27
0.0525	40	1.0 " "	45					0.55
0.082	25	1.0 " "	46					0.75
0.150	40	1.0 " "	45					1.98
0.033	25	0.5 Glyceryl triacetate.	26					0.37
0.0525	40	0.5 " "	45					0.73
0.082	25	0.5 " "	46					1.00
0.150	40	0.5 " "	45					2.58
Alanine.								
0.045	40	1.0 Ethyl butyrate.	24		0.23	0.21	0.42	0.31
0.16	40	1.0 " "	26	0.32		0.38		0.27
0.045	40	0.5 Glyceryl triacetate.	24		0	0.06	0.37	0.35
0.16	40	0.5 " "	26	0		0.21		0.25
Leucine.								
0.045	40	1.0 Ethyl butyrate.	24		0.36	0.35	0.20	0.36
0.10	40	1.0 " "	24	0.29		0.11	0.15	0.51
0.16	40	1.0 " "	26	0.20		0.33		0.43
0.10	40	0.5 Glyceryl triacetate.	24	0.22		0.35		0.62
0.16	40	0.5 " "	26	0.28	0	0.16		0.48
Phenylalanine.								
0.045	40	1.0 Ethyl butyrate.	25		0.04	0.14	0.08	0.38
0.10	40	1.0 " "	25	0.23	0.08	0.07	0.10	0.39
0.045	40	0.5 Glyceryl triacetate.	25		0	0.10	0.13	0.49
0.10	40	0.5 " "	25	0.20	0	0.08	0.27	0.41
Tyrosine.								
0.045	40	1.0 Ethyl butyrate.	24		0.17	0.15	0.31	0.28
0.045	40	0.5 Glyceryl triacetate.	24		0	0.08	0.27	0.41

results of Table II, and only in so far as they relate to the present subject, will be given. Considering the amino-acids alone first, it will be noticed that as a rule the action is as marked toward ethyl butyrate as it is toward glyceryl triacetate. Omitting glycine, this is seen to be true throughout, and further, that the action even at $C_H = 10^{-9.0}$ is comparatively small and apparently independent of the concentration of the amino-acids. The ir-

regularities of the results are due to the relatively large experimental errors.

With regard to glycine,[12] the first point to be brought out is that the ratios of the actions toward glyceryl triacetate and ethyl butyrate ranged from 1.30 to 1.37, mean 1.33. For the other amino-acids the ratio is also not very far from unity. This contrasts sharply with the ratios of the actions of the dipeptides which ranged from 5 to 12, mean 8.3 This proves that the actions are not due to the hydroxyl ion concentrations, but that the amino-acids and peptides are the important factor. The hydrolytic action of 1 gm. molecule of glycine may be calculated just as with the peptides. Toward ethyl butyrate for 26 hours' action it is found to be 6.1×10^2; for 45 to 46 hours 8.2×10^2 (mean); toward glyceryl triacetate it is found to be 8.4×10^2 for 26 hours' action, and 10.8×10^2 for 45 to 46 hours. With dipeptides the mean actions found for 19 hours were 6.2×10^2 toward ethyl butyrate and 46.4×10^2 toward glyceryl triacetate. This indicates that the action of the dipeptides toward the ethyl butyrate is due mainly to the amino and carboxyl groups and confirms the results obtained with the glycylglycine ester hydrogen halides. Subtraction of the amino-acid glyceryl triacetate value from the value for the peptide leaves an action of 35.8×10^2 to be accounted for by the group $-CO-NH-$ or $-C(OH)=N-$. The mean value found with the peptide ester hydrogen halide was 17.2×10^2 but the difference as already stated may well be due to the shift in the tautomeric equilibrium as the solution became neutral.

The action of glycine ethyl ester hydrochloride was also studied and the following results were obtained.

0.10 gm. of glycine ethyl ester hydrochloride, 25 cc. of water, 23 hours' action, 1.0 cc. of ethyl butyrate, or 0.5 cc. of glyceryl triacetate.

pH	6.0	7.0	8.0	9.0
Ethyl butyrate	0.04 (5.0)	0.04 (6.0)	0.09 (6.0)	0.11 (7.0)
Glyceryl triacetate	0 (5.0)	0.05 (5.8)	0.10 (5.5)	0.20 (6.0)

[12] The glycine solutions were made up according to Sörensen's directions for buffer mixtures and contained sodium chloride.

Just as with the glycylglycine ester hydrogen halides the solution became neutral fairly rapidly. The very slight action when the amino-acid carboxyl groups are masked is also significant.

The actions of the simpler amino-acids toward different esters were treated in detail by Hamlin and it was shown by him that if the esters are arranged in a series according to the extent of their hydrolyses, different arrangements result with the different amino-acids for the same hydrogen ion concentration and different as well from an isohydric acid solution containing no amino-acid.

Since the keto-lactam group is present in other substances besides peptides, some experiments were made to find whether these exerted any hydrolytic action on esters in slightly alkaline solutions. Urea, studied at hydrogen ion concentrations between $10^{-4.0}$ and $10^{-10.0}$ gave no action whatsoever on ethyl butyrate or glyceryl triacetate. Hippuric acid showed practically no action on ethyl butyrate and very slight action on glyceryl triacetate at $H^+ = 10^{-9.0}$, but the solutions rapidly became neutral or slightly acid. The hippuric acid exerted very little buffer action. A series of experiments was therefore run in which Sörensen's glycine buffer mixture for $C_H = 10^{-9.0}$ was added to the solution. The results follow.

Hippuric acid.	Glycine.	Volume.	Time of action.	Action.	
				Ethyl butyrate.	Glyceryl triacetate.
gm.	*gm.*	*cc.*	*hrs.*		
0.10	0.033	25	26	0.44(6.0)	0.60(5.8)
0.10	—	25	26	0 (6.0)	0.04(5.5)
—	0.033	25	26	0.27(6.5)	0.37(5.5)
0.20	0.082	25	46	0.66(7.0)	1.14(6.0)
0.20	—	25	46	0 (6.0)	0.10(6.0)
—	0.082	25	46	0.60(7.0)	0.80(5.5)

The action of the hippuric acid alone calculated from these results is 0.17 and 0.06 on the ethyl butyrate and 0.19 and 0.24 on the glyceryl triacetate; small actions it is true, but distinct.

These results indicate that the structure of the compound as a whole is of importance in determining the equilibrium between the tautomeric forms, if these be involved in the actions. This question will be taken up again later.

Action of Imido Ester on Esters.

In order to obtain further evidence with regard to the hydrolytic action on esters of substances containing the enol-lactim grouping, the behavior of an imido ester was studied. Imido

TABLE III.

Hydrolytic Actions of Ethyl Imidobenzoate on Esters.

Substance.*	Time of action.	Initial pH.	Blanks. Immediate titration. Direct + formol.	Blanks. Final titration. Direct + formol.	pH.	Actions. 1.0 cc. ethyl butyrate.	pH.	Actions. 0.5 cc. glyceryl triacetate.	pH.
gm.	*hrs.*								
0.075	23	6.0	2.39+0.19	0.12+2.27	(8.0)	0.15	(6.5)	0.71	(6.5)
0.075	23	7.0	0.64+0.04	0 +0.47	(8.5)	0.20	(7.5)	1.39	(6.5)
0.075	23	8.0	0.11+0.11	0 +0.17	(8.5)	0.05	(7.5)	1.51	(7.0)
0.075	23	9.0	−0.04+0.15	0 +0.10	(8.5)	0.08	(7.5)	1.34	(7.0)
0.06	22	4.0	2.87+0	0.33+2.66	(3.8)	0.21	(3.8)	0.28	(3.8)
0.06	22	6.0	1.61+0	0.02+1.60	(8.5)	0.10	(6.5)	0.62	(6.0)
0.06	22	8.0	0.10+0	0 +0	(9.0)	0.24	(7.0)	1.56	(6.8)
0.06	22	10.0	−0.10+0	0 +0.14	(9.0)	0	(7.0)	1.12	(6.5)
0.10	22	7.0	1.04+0	0 +0.31	(9.0)	0.61	(7.5)	1.89	(7.5)
0.05	4	7.0	0.51+0	0.06+0.34	(7.8)			0.27	(7.2)
0.05	22	7.0	0.51+0	0 +0.34	(9.5)			1.18	(7.0)
0.05	47	7.0	0.51+0	0 +0.36	(9.5)			1.53	(5.8)

0.05 gm. portions in solution heated in incubator (38°) for different lengths of time after being brought to pH = 7.0, then tested at once and also after neutralization.

Heated 4 hrs.	20	7.8	0.04+0.24	0 +0.43	(8.5)			1.09	(7.0)
" 4 "	20	7.0	0.26+0.28	0 +0.40	(8.5)			1.22	(6.8)
" 23 "	23	10.0	−0.16+0.30	0.04+0.34	(8.0)			0.09	(6.0)
" 23 "	23	7.0	0.08+0.34	0.04+0.39	(7.8)			0.22	(5.8)
" 48 "	22	10.0	−0.14+0.35	0.04+0.24	(7.6)			0	(5.5)
" 48 "	22	7.0	0.04+0.26	0.04+0.28	(7.0)			0	(5.0)
Boiled 5 min.	20	9.0	−0.24+0.26	0.04+0.33	(7.8)	0	(6.0)	0.06	(5.5)
" 5 "	20	7.0	0.08+0.30	0.04+0.46	(8.0)	0	(6.0)	0.27	(5.5)

TABLE III—*Concluded.*

0.055 gm. of ethyl imidobenzoate hydrochloride, 25 cc. of water, 20 hours, action, pH = 7.0 initially. Immediate titration 0.27 + 0; titration after standing 0 + 0.37 (pH = 8.0) for blanks.

Ester.	Action.	Ester.	Action.
cc.		*cc.*	
0.5 Methyl acetate.	0.94 (7.5)	0.5 Ethyl benzoate.	0.03 (7.8)
0.5 Ethyl "	0.70 (7.0)	0.5 (gm.) Phenyl benzoate.	0 (8.5)
0.5 Phenyl "	0.62 (7.0)	0.5 Ethyl butyrate.	0.20 (7.8)
0.5 Glyceryl triacetate.	1.39 (7.0)	1.0 Olive oil.	0.16 (9.0)
0.5 Methyl benzoate.	0.09 (8.0)	1.0 Cottonseed oil.	0.04 (9.0)

* Weighed as ethyl imidobenzoate hydrochloride.

esters possess the general formula $\mathrm{R-C}\begin{array}{l}\diagup \mathrm{O\ R'}\\ \diagdown \mathrm{N\ R''}\end{array}$. One of the simplest members of this class, and the one most readily prepared (in the form of hydrochloride) is ethyl imidobenzoate, $\mathrm{C_6H_5-C}\begin{array}{l}\diagup \mathrm{OC_2H_5}\\ \diagdown \mathrm{NH}\end{array}$. The results obtained with this substance will be presented first in tabular form (in Table III) and then their significance will be discussed. The volume of the solution in each experiment was 25 cc.

Ethyl imidobenzoate decomposes in aqueous solution, the decomposition being accelerated by acids or alkalis.[13] In this decomposition ethyl benzoate, benzamide, benzonitrile, and ammonia or ammonium chloride may be formed. The decomposition is shown by the titration values of the blanks in the table. The column headed "Immediate titration" gives the results for titration to the first pink color with phenolphthalein without ("Direct") and with ("Formol") addition of neutralized formaldehyde solution to the solutions as first prepared. It is seen that in every case during the time of the action the direct and formol titration values have become practically interchanged. Because of the possibility of the formation of different products in the reaction, it is impossible to state the substances present at the different times. This makes it difficult to determine the ester blanks to be used as corrections. The experiments with

[13] Stieglitz, J., *Am. Chem. J.*, 1908, xxxix, 29 and 166.

ethyl benzoate show that the latter was not hydrolyzed in the reactions. Series of experiments with benzamide and with benzonitrile starting with $C_H = 10^{-6.0}$ to $10^{-9.0}$ showed no hydrolytic action whatsoever toward ethyl butyrate or glyceryl triacetate. A series of experiments with ammonium chloride gave the following results. 0.025 gm. of NH_4Cl, 25 cc. of H_2O, 19 hours' action.

pH	6.0	7.0	8.0	9.0
Ethyl butyrate	0.08(5.5)	0.13(6.8)	0.15(6.0)	0.18(7.8)
Glyceryl triacetate	0.12(5.0)	0.17(5.0)	0.22(6.0)	0.46(5.5)

These values were used throughout Table III as the ester corrections. The choice may appear to be somewhat arbitrary, but these values are if anything greater than those actually occurring, since in the imido ester experiments the ammonium chloride was probably not all present from the beginning. At any rate, this question is of minor importance when compared with the magnitude of the actions themselves.

The action toward ethyl butyrate was found to be small except in one experiment. The actions toward glyceryl triacetate were comparatively large, however. A maximum action was observable at the hydrogen ion concentration of $C_H = 10^{-8.0}$, compared with more acid or more alkaline solutions. This may well be due to the more rapid decomposition of the imido ester in the latter solutions. With different amounts of imido ester and for different periods of time, no simple proportionality with the amount of hydrolysis is noticeable. This may also have been due to the decomposition of the imido ester. The action was also lost when the imido ester in neutral aqueous solution was allowed to stand at 38° for 23 hours or was boiled for 5 minutes. These experiments showed that the alkalinity of the solutions was of secondary importance in the actions.

The results obtained with the different esters may be calculated to correspond to the actions which would be obtained with 1 gm. equivalent of the ester under the same conditions. The following values $\times 10^{-2}$ are obtained in this way.

Glyceryl triacetate.	1.77	Ethyl acetate.	1.34	Ethyl benzoate.	Trace.
Phenyl acetate.	1.62	" butyrate.	0.53	Phenyl "	"
Methyl "	1.45	Methyl benzoate.	0.23		

This order of decreasing actions for ethyl imidobenzoate at $C_H = 10^{-7.0}$ is the same as that for glycylglycine at $C_H = 10^{-9.0}$ except that the positions of the first two members, glyceryl triacetate and phenyl acetate, are interchanged. This shows a marked similarity in behavior, while the minor difference may be due to secondary difference in structure.

The action of 1 gm. equivalent of the imidobenzoate on glyceryl triacetate (0.5 cc.+), 22 hours, $C_H = 10^{-7.0}$ may also be given (mean values are shown).

C_H	$10^{-4.0}$	$10^{-6.0}$	$10^{-7.0}$	$10^{-8.0}$	$10^{-9.0}$	$10^{-10.0}$
Action	8.7	18.4	37.8	42.8	33.2	34.6

The results, especially for $C_H = 10^{-7.0}$ to $10^{-10.0}$ are not far removed from the results for the dipeptides at $C_H = 10^{-9.0}$. The decomposition of the imido ester must also be taken into account here. The maximum action at $C_H = 10^{-8.0}$ is also evident. Since the action toward ethyl butyrate is small or negligible, it is evident that the actions observed confirm the view based upon the behavior of the dipeptides, that the action is due to the grouping $-C(OR)=N-$.

Without considering the possible mechanism or cause for the reactions in either case, the ester-hydrolyzing action of the imido ester is similar to that of the naturally occurring lipases in that a maximum action is obtained at a definite hydrogen ion concentration, and that the activity of both is destroyed by the action of acids, of alkalis, standing in solution, and heating in solution.

Action of Alkali on Proteins.

In order to determine whether or not the conditions which are known to favor the enol-lactim grouping in simple substances will produce ester-hydrolyzing groups or substances from proteins, a number of experiments were carried out in which casein, gelatin, and castor bean globulin were treated with alkali of different strengths and after neutralization tested for ester-hydrolyzing action. A typical experiment may be described as follows.

Three 2 gm. portions of casein (Kahlbaum's) were treated with 25 cc. of sodium hydroxide solution of the following concentrations: (*a*) 3.0 molar, (*b*) 1.5 molar, (*c*) 0.6 molar. They were thoroughly shaken, and allowed to stand for 24 hours at room temperature. The mixtures then had the appearance of homogeneous light yellowish brown solutions. They were diluted with 75 cc. of water each, neutralized with concentrated hydrochloric acid to about $C_H = 10^{-9.0}$, and dialyzed in collodion bags for 19 hours against running water.

(*a*) After dialysis, the volume had increased to 310 cc., a small amount of solid was present, $C_H = 10^{-7.8}$. It was brought to $C_H = 10^{-7.0}$, and 45 cc. portions were tested.

(*b*) Volume, 395 cc. after dialysis, turbid, $C_H = 10^{-8.0}$. It was brought to $C_H = 10^{-7.0}$, and 50 cc. portions were tested.

(*c*) Volume increased to 400 cc., slightly turbid, $C_H = 10^{-9.0}$. It was brought to $C_H = 10^{-7.0}$, and 50 cc. portions were tested. Actions (corrected for blanks), 47 hours, (*a*) Ethyl butyrate 0.08; Glyceryl triacetate, 0.56; (*b*) Ethyl butyrate 0.11; Glyceryl triacetate 0.50; (*c*) Ethyl butyrate 0.10; Glyceryl triacetate 0.48.

In some experiments the alkali was removed completely by dialysis; in others, it was neutralized by acid without dialysis; in others, removed in part first by dialysis and then neutralized, and *vice versa*. Different strengths of alkali were used for various periods of time. In about half of the experiments toluene was added throughout in every preparation: in the remainder no toluene at all was added. No difference in results was obtained in the two series, and no growth was obtained either in agar or in blood agar. Some of the results obtained are summarized in Table IV.

In the titrations with the gelatin experiments it was necessary to use the formol method in order to obtain satisfactory end-points. With the casein and castor bean globulin titrations formaldehyde was not added. The effect of boiling the alkali-treated protein solution before testing the actions was tried several times. With the casein mixtures no difference in action was observed between the heated and unheated mixtures; with the gelatin, in one case two-thirds of the activity was lost; in two other attempts there was no change.[14] On boiling these solutions there was a small increase in alkalinity, greater with casein than

[14] Reference may be made to the thermostabile lipase described by Kendal, A. I., Walker, A. W., and Day, A. A., *J. Infect. Dis.*, 1914, xv, 455.

TABLE IV.

Hydrolytic Actions of Alkali-Treated Proteins on Esters.

Treatment.					Action.				
	NaOH solution.								
Protein.	Concentration.	Amount.	Time standing.	Method of neutralization.	pH.	Time of action.	Original protein tested.	Ethyl butyrate.	Glyceryl triacetate.
Casein.									
gm.	N	*cc.*	*hrs.*			*hrs.*	*gm.*		
2.0	0.11	15	24	Diluted, dialyzed.	7.5	45	0.35	0.03	0.87
2.0	2.0	25	22	Diluted, neutralized, dialyzed, neutralized.	7.0	46	0.25	0.05	0.37
2.0	1.0	25	22	"	7.0	46	0.30	0.08	0.48
2.0	0.5	25	22	"	7.0	46	0.30	0.06	0.55
2.0	1.0	25	48	Diluted, neutralized, to:	4.0	25	0.50		0.15
2.0	1.0	25	48	"	6.0	25	0.50		0.10
2.0	1.0	25	48	"	8.0	25	0.50		1.17
2.0	1.0	25	48	"	10.0	25	0.50		1.47
2.0	1.0	25	24	"	8.0	22	0.40		1.68
2.0	1.0	25	24	"	7.0	22	0.40		0.91
2.0	1.0	25	18	"	8.0	24	0.24		1.13
2.0	1.0	25	24	"	8.0	22	0.53		2.00
2.0	1.0	25	24	"	8.0	22	0.50		1.73
5.0	0.4	30	4	"	8.0	18	1.0		1.86
5.0	0.4	30	4	"	8.0	18	0.25		0.74
Gelatin.									
2.0	1.0	25	45	"	7.0	46	0.40	0.13	0.96
4.0	1.0	50	72	"	8.0	42	0.40	0.22	1.80
4.0	1.0	50	72	"	7.0	42	0.25		0.86
2.0	0.1	50	45	"	8.0	45	0.40		0.63
2.0	0.2	50	45	"	8.0	45	0.40		1.41
2.0	0.5	50	45	"	8.0	45	0.40		2.38
2.0	1.0	50	45	"	8.0	45	0.40		4.28
Castor bean globulin.									
1.0	2.5	25	24	Diluted, dialyzed.	8.5	48	0.20	0.24	0.73
1.0	2.5	25	24	Neutralized, diluted, dialyzed.	7.0	22	0.15	0.09	0.27

with gelatin. Dialysis removed or destroyed the activity of the gelatin preparations but only decreased that of the casein preparations somewhat. Dissolving the gelatin in water, bringing the solutions to the hydrogen ion concentrations $10^{-6.0}$ to $10^{-9.0}$, and testing their actions toward ethyl butyrate and glyceryl triacetate gave only a trace of hydrolysis. A parallelism was observed between the action of the gelatin preparation toward the glyceryl triacetate and the amount of formol titration. From the evidence presented with the peptides and the amino-acids, the amount of amino carboxyl groups appears to have no direct connection with the amount of glyceryl triacetate hydrolysis, so that this does not mean that the action observed here was due to the presence of peptides.

The main point brought out in these results is the very marked hydrolytic actions of proteins after treatment with alkali. Not enough work was done on the question to show the dependence of the action on the method and details of treatment, on a number of different esters, etc. Dialysis of the casein and castor bean globulin preparation indicates that simple substances are not responsible for the actions. A systematic study of these relations is now being carried on in this laboratory by Dr. Florence Hulton Frankel.

DISCUSSION.

The experimental evidence presented in the first part of this paper led to the view that the inactivation of lipase was due to a tautomeric change or rearrangement within the molecule. Conversely, therefore, active lipase material should be formed by a tautomeric change in which the equilibrium between the tautomeric substances would be such that the lipolytically active structure was capable of existing. The lipase materials prepared from castor beans and soy beans in the course of this work were essentially protein in character, and the most obvious grouping having such possibilities of tautomerism is that involved in the peptide linking. The next step in the work was therefore to find whether such tautomeric groupings show hydrolytic actions toward ester and whether these actions were lost by conversion into the keto-lactam structures. The work described with the peptides, amino-acids, and imido ester showed that the

grouping $-C(OR)=N-$, in which R may stand for hydrogen or an organic radical, has hydrolytic action on esters and that the tautomeric group $-CO-NR-$ does not. This evidence does not show anything with regard to the active lipolytic grouping in enzymes, but the work with the action of alkalis on protein to form ester-hydrolyzing substances under conditions favoring an enol-lactim structure may be considered to be evidence bearing on this point.

It must be emphasized that no direct conclusive evidence is presented as to the actual chemical configuration of the active lipase grouping. The steps in the reasoning may be summarized as follows.

Inactivation (and therefore also activation) is assumed to be due to a tautomeric rearrangement whose possible nature is indicated. Simple substances possessing such structures show the actions and some other properties of naturally occurring lipases present in protein materials. Inactive proteins treated in such a way as to produce the supposedly active grouping show ester-hydrolyzing properties.

Whether it is possible to go much beyond this in the present state of the knowledge of the chemical nature of proteins and the changes they undergo with simple treatment is an open question. However, one possible line of development bearing upon the present problem may be indicated. The equilibrium in solution between the tautomeric forms of ethyl acetate depends to a great extent upon the solvent.[15] This suggests that with the enol-lactim keto-lactam tautomerism in proteins the colloidal properties of the protein material may well exert an influence on the grouping comparable to the effects of solvents, and that the decreased stability or increased rates of inactivation of enzyme preparations, when separated to a greater or less extent from colloidal and other matter not connected with the actions, may be placed in parallel with the actions of the solvents on the equilibria between the tautomeric forms of ethyl acetacetate, etc.

Reference may be made to some preliminary tests in this connection. Precipitation and adsorption of the proteins of the esterase and lipase preparations by aluminium hydroxide (alumina

[15] Meyer, K. H., and Willson, F. G., *Ber. chem. Ges.*, 1914, xlvii, 832.

cream) showed that under certain conditions the activities of these substances were retained somewhat longer than in the absence of aluminium hydroxide. Attempts to adsorb dipeptides in the tautomeric enol-lactim form on egg albumin or gum arabic and then to neutralize the solutions and still retain the former in the active ester-hydrolyzing form failed.

In the development of the hypothesis regarding the active grouping in lipase actions, the experimental work and discussion were limited almost entirely to the peptide linking. It is evident, however, that such tautomeric structures, enol-lactim and keto-lactam, may be present in other groupings, and the results of this investigation in no way limit the lipolytic activity to the peptide linking. In view of the complexity of the protein molecule, it is highly probable that such similar tautomeric groups may be present in combination with other groups and that the specificities of the actions are in part dependent upon these. This is especially true of the actions described with the esterase preparation, in which there was marked activity toward ethyl butyrate. This action may be due to the free amino carboxyl groups under favorable conditions as shown by glycine, and also to the enol-lactim structure in suitable combination.

It must be admitted that the treatment of proteins with alkali to form active substances is rather strenuous. Unquestionably, simpler methods, comparable to those taking place in nature, will be found to produce the same effects. The fact that dilute alkalis inactivate the castor bean globulin lipase, while a certain higher concentration of alkali produces an ester-hydrolyzing substance from the inactive globulin preparation, indicates that differently placed groups in the molecule are involved in these two changes.

SUMMARY.

The inactivation of esterase and lipase preparations by acids, bases, neutral salts, alcohols, acetone, esters, and heat, led to the hypothesis that the active enzyme grouping in these substances possessed the enol-lactim structure, $-C(OH) = N-$, which became inactive by tautomerization to the keto-lactam structure, $-CO-NH-$.

This hypothesis was tested by studying the actions of such groupings in dipeptides and an imido ester. The dependence of the actions on the grouping was discussed, and certain similarities were pointed out between the behavior of the imido ester and naturally occurring lipases.

The production of ester-hydrolyzing substances by the action of alkali on proteins under conditions which might be expected to form the hypothetical active grouping was shown.

The bearing of this work on the nature of the chemical group responsible for lipase actions was discussed.

THE PHYSIOLOGICAL BEHAVIOR OF RAFFINOSE.

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Though raffinase is frequently found in plants, fungi, bacteria, yeast, and invertebrate animals (1, 2, 3, 4), its presence in the body in higher animals has never been positively demonstrated. The blood serum, bile, extracts of the mucous membrane of the stomach and small intestine, pancreas, thyroid, and testicle have no raffinose-splitting power (5, 6, 7). After injecting 11 to 22 gm. of raffinose subcutaneously in man, F. Voit recovered 65 to 92 per cent of it in the urine (8). In Magnus-Levy's rabbit experiments, however, subcutaneously injected raffinose was recovered quantitatively in the urine (9). After feeding 10 gm. of raffinose to starving hens, Külz noted some glycogen formation in the liver (10). Discussing Külz's results, however, Pflüger considered the glycogen formation after raffinose feeding very doubtful (11). When raffinose was fed to a diabetic dog, severe diarrhea followed. An increase of urine sugar was rather uncertain (12). Comparing the rapidity of absorption of various kinds of sugar administered into intestinal loops of rabbits, Hédon demonstrated that raffinose is by far more slowly absorbed than the other sugars (13). Halász injected 49 to 147 gm. of raffinose into the rectum, in men, and examined the feces, eliminated from 2 to 6¼ hours later, for raffinose and its cleavage products. 2.6 to 74.7 gm. of raffinose disappeared (14).

We have attempted to obtain additional evidence concerning the physiological behavior of raffinose. Part of this sugar used was obtained through the courtesy of Dr. C. S. Hudson in Washington; the rest was a Kahlbaum preparation.

The Influence of the Hydrogen Ion Concentration of the Medium upon the Activity of Raffinase.

As a preliminary to renewed search for raffinase in the animal body, it became essential to learn what medium is favorable for the action of this enzyme.

Methods.—The hydrogen ion concentration of the medium was determined by the indicator method. The standard solutions employed were mono- and dipotassium phosphate mixture, acetic acid and sodium acetate mixture, and hydrochloric acid and disodium citrate mixture. Phenolphthalein, neutral red, methyl red, Congo red, and tropeolin 00 were used as indicators (15, 16, 17). Raffinase solution was prepared from brewers' bottom yeast by the method suggested by Hudson for sucrase preparation (10 day autolysis and 3 day dialysis) (18). The clear enzyme solution, preserved with chloroform, was neutral and contained 42 mg. of nitrogen per 100 cc. It gave a positive biuret reaction. No melibiase was present.

To test the enzyme action in media with a desired hydrogen ion concentration, either the standard regulator mixtures or sulfuric acid, diluted in various degrees, were used. The latter were prepared by diluting 0.2, 2, 4, 10, 20, 30, 40, and 50 cc. respectively of 0.01 N sulfuric acid to 100 cc. with distilled water. The test was completed as follows: 15 cc. of a regulator mixture or a diluted sulfuric acid and 3 cc. of a 10 per cent raffinose solution were heated in a stoppered bottle in a thermostat at 40°C. 2 cc. of the raffinase solution were then added. After 40 minutes 1 cc. of a 3 per cent mercuric chloride solution was added, in order to stop the enzyme action instantly. The bottle was cooled, and after 4 hours, after which any multirotation of the cleavage products might be avoided, the filtrate of the mixture was examined polarimetrically. The temperature of the thermostat during the experiment did not vary more than 0.5°C. Another portion of the same mixture of the standard solution or diluted sulfuric acid with raffinose and enzyme solution was used for determining the hydrogen ion concentration. The mixtures showed the same hydrogen ion concentration before or after heating. When a standard solution was mixed with raffinose and enzyme, the original hydrogen ion concentration was unchanged. In case of diluted sulfuric acid, the hydrogen ion concentration was markedly decreased by the addition of raffinose solution. In calculating the extent of the inversion of raffinose, a decrease of rotation to half of the original was considered to show the complete inversion of raffinose into levulose and melibiose. In order to see if the acidity itself was adequate to invert raffinose, the enzyme preparation was previously boiled for 1 hour in some experiments.

From Table I and Fig. 1 it will be seen that under the experimental conditions employed, the optimal zone of hydrogen ion concentration of the medium for the raffinase activity is pH 3.8–5.4. The same hydrogen ion concentration, no matter how it is prepared, shows the same influence upon the raffinase activity. The most favorable hydrogen ion concentration for the activity of sucrase is reported to be pH 4.4–4.6 (15) or pH 5.25–3.67 (19). Raffinase, which was formerly considered to be identical with sucrase, has nearly the same relation to hydrogen ion concentration of the medium.

TABLE I.

The Influence of the Hydrogen Ion Concentration of the Medium upon the Activity of Raffinase.

Medium.	pH.	Decrease of rotation.	Inversion of raffinose.
Experiment I.			
		Ventzke°	*per cent*
Phosphate mixture................	8.7	0	0
" "	8.0	0	0
Acetate "	6.7	1.05	24.3
" "	5.3	1.75	40.5
" "	4.3	1.90	43.9
" "	3.1	1.40	32.4
Citrate "	2.2	0.10	2.3
" " (with boiled enzyme)........................	2.2	0	0
Experiment II.			
Phosphate mixture................	8.7	0	0
" "	8.0	0	0
" "	7.4	0	0
Acetate "	6.7	1.05	24.3
" "	6.0	2.00	46.2
" "	5.3	2.05	47.4
" "	4.3	2.00	46.2
" "	3.4	1.85	42.8
Citrate "	2.2	0	0
" " (with boiled enzyme)........................	2.2	0	0
Experiment III.			
Sulfuric acid........................	6.8	0.90	20.8
" "	6.5	1.45	33.5
" "	6.1	1.75	40.5
" "	4.9	2.15	49.7
" "	4.0	2.05	47.4
" "	3.5	2.00	46.2
" "	3.0	1.35	31.2
" "	2.5	0.90	20.8
" " (with boiled enzyme)..	2.5	0	0
Experiment IV.			
Sulfuric acid........................	6.8	0.65	15.0
" "	6.5	1.19	27.5
" "	6.1	1.59	36.8
" "	4.9	1.91	44.2
" "	4.0	1.90	43.9
" "	3.5	1.55	35.8
" "	3.0	1.20	27.7
" "	2.5	0.55	12.7
" " (with boiled enzyme).	2.5	0	0

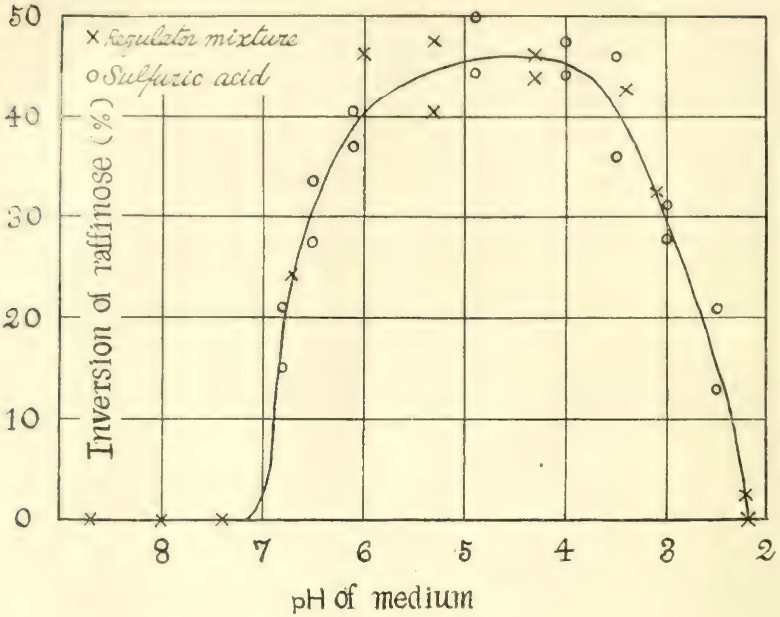

Fig. 1.

Does Raffinase Occur in the Alimentary Tract?

Pautz and Vogel (5) and Fischer and Niebel (6) failed to detect raffinase in the body of higher animals. We have examined (*a*) human saliva, (*b*) bladder bile of a rabbit, (*c*) water extract of dog pancreas, (*d*) water extracts of the liver of a dog and a rabbit, and (*e*) water extracts of the mucous membrane of the small and large intestine of a dog and a rabbit.

Methods.—The organs were comminuted with double volumes of water and kept for 24 hours with toluene at room temperature. The mixture was then filtered with cloth. When the filtrate was strongly acid, sodium carbonate solution was carefully added so that the final reaction was very slightly acid. 5 cc. of the filtrate were mixed with 1 cc. of a 10 per cent raffinose solution and 0.2 cc. of toluene, and incubated for 24 hours at 38–40°C. The mixture was then diluted with 19 cc. of water and clarified with 5 cc. of colloidal iron solution. The final water clear solution was examined for its rotation and reducing power. For control, another sample of the same mixture was examined without incubation. Saliva and bile were tested in the same manner, but here the examination was performed

both at the original reaction of these fluids and in a condition slightly acidified with dilute acetic acid. The saliva and pancreas extract employed were strongly amylolytic, and the extract of the mucous membrane of the small intestine contained sucrase.

The experiments all failed to show the presence of raffinase.

It has been reported that inulin, sucrose, and erythrodextrin can be inverted in the stomach, not by corresponding enzymes, but by the free hydrochloric acid of the stomach juice (20, 21, 22, 23). To test the possibility of raffinose being split in this way, media containing 1 per cent raffinose and 0.04, 0.1, or 0.2 per cent hydrochloric acid were incubated for $5\frac{1}{2}$ hours at 38–40° C. and then examined polarimetrically at once.

TABLE II.

The Inversion of Raffinose in Media Containing 0.04, 0.1, or 0.2 per cent Hydrochloric Acid.

HCl in medium.	Examined immediately.		Examined after incubating $5\frac{1}{2}$ hrs. at 38–40°C.	
	Rotation.	Reduction.	Rotation.	Reduction.
per cent	*V°.*		*V°.*	
	+5.90	−	+4.30	+++
0.2	+5.90	−	+4.70	+++
	+5.90	−	+4.60	+++
0.1	+5.85	−	+4.83	+++
			+4.83	+++
0.04	+5.85	−	+5.38	++
			+5.35	++

From Table II it will be seen that the inversion of raffinose in the stomach is possible under suitable conditions.

The Activity of Raffinose in Rabbit Serum.

From the results shown in Table I it will be seen that the normal reaction of the blood is unfavorable for the activity of raffinase (dog blood pH 7.32–7.64, rabbit blood 7.18–7.70) (24). To ascertain whether the parenteral administration of raffinose can call forth the corresponding enzyme in the blood, it was necessary to determine how raffinase acts in the serum.

Methods.—The blood was taken from a jugular vein and cooled 3 hours, after which the serum was separated by centrifuging. A mixture was prepared as follows: 3 cc. of serum (or 3 cc. of 0.9 per cent NaCl) + 1 cc. of 10 per cent raffinose + 1 cc. of raffinase solution (the original concentration is designated as "raffinose stronger," a five times diluted solution as "raffinose weaker") + 1 cc. of water (or 1 cc. of 0.025 N CH_3COOH—"acid weaker," or 1 cc. of 0.075 N CH_3COOH—"acid stronger," or 1 cc. of 0.025 N NaOH—"alkali") + 0.2 cc. of toluene. The mixture was incubated for 40 hours at 38–40°C., and then diluted with 19 cc. of water. After clarifying with 5 cc. of colloidal iron solution, the water clear filtrate was examined for rotation (Ventzke degrees with a 2 dm. tube) and reducing power.

TABLE III.

The Activity of Raffinase in Rabbit Serum.

Raffinose plus			Reaction (litmus).	Rotation.	Reduction.
Serum or saline solution.	Raffinase.	Acid, alkali, or water.			
				V°.	
Serum.	Raffinase (weaker).	Water.	Alkaline.	+1.95	−
"	"	Acid (weaker).	"	+1.91	±
"	"	Alkali.	"	+1.92	−
Saline solution.	"	Water.	Neutral.	+1.02	+++
" "	"	Acid (weaker).	Acid.	+1.02	+++
" "	"	Alkali.	Alkaline.	+1.91	−
Serum.	Raffinase (boiled).	Water.	Neutral.	+1.92	−
Saline solution.	"	"	"	+1.91	−
Serum.	Raffinase (stronger.)	Water.	Alkaline.	+1.67	++
"	"	Acid (weaker).	"	+1.05	+++
"	"	" (stronger).	Neutral.	+1.05	+++
Saline solution.	"	" (weaker).	Acid.	+1.90	±
" "	"	" (stronger).	"	+1.90	+
" "	"	Water.	Neutral.	+1.03	+++
Serum.	Raffinase (boiled).	Acid (stronger).	"	+1.92	−

From Table III it will be seen that the reaction of the serum is very unfavorable for the activity of raffinase. The enzyme shows its function only when there is an abundance of raffinase and especially when the mixture is slightly acidified.

In a few experiments the enzyme preparation was injected

intravenously into rabbits and after a certain interval the serum was taken from the animal and examined for raffinase.

Methods.—The raffinase solution, made isotonic with sodium chloride and freed from chloroform by a current of air, was injected into an ear vein. 15 to 30 minutes later, the blood sample was taken from a jugular vein. Serum separation and 24 hour digestion were performed as described on p. 130. The mixture for digestion was 3 cc. of serum + 5 cc. of 10 per cent raffinose + 1 cc. of water (or 1 cc. of 0.075 N CH_3COOH) + 0.2 cc. of toluene.

TABLE IV.

The Raffinose-Splitting Power of the Serum after Intravenous Injection of Raffinase.

Rabbit.			No.	I.		II
Rabbit.			Body weight, *kg.*	1.80		2.16
Raffinase solution into ear vein.			Volume, *cc.*	25		35
Raffinase solution into ear vein.			Duration of injection, *min.*	3		4
Interval between raffinase injection and blood taking, *min.*				15	30	15
Serum test.	Examined immediately.		Rotation, $V°$	+9.80	+9.80	+9.90
Serum test.	Examined immediately.		Reduction	–	–	–
Serum test.	Examined after 24 hrs.	Without acid.	Rotation, $V°$	+9.50	+9.80	+8.20
Serum test.	Examined after 24 hrs.	Without acid.	Reduction	+	–	+++
Serum test.	Examined after 24 hrs.	With acid.	Rotation, $V°$			+5.90
Serum test.	Examined after 24 hrs.	With acid.	Reduction			+++

From Table IV it will be seen that the activity of raffinase injected intravenously is maintained for a short period only. Disappearance of its activity may be due to decomposition or elimination of the enzyme from the circulation.

The Utilization of Raffinose Parenterally Administered into Rabbits. The Raffinose-Splitting Power of the Serum of the Same Animals.

From the reports of Mendel and Kleiner (25), Hogan (26), Kuriyama (27), and others, it appears that after parenteral administration sugars such as sucrose and lactose, which are foreign

TABLE V.

The Utilization of Raffinose, Injected Parenterally into Rabbits. The Raffinose-Splitting Power of the Serum of the Same Animals.

Rabbit.	Date.	Administration of raffinose (ro) or raffinase(ra) + raffinose (ro).	Urine.			Serum.					
						Examined immediately.		Examined after incubating 48 hrs. at 38–40°C.			
								Without acid.		With acid.	
			Volume.	Raffinose.	Total raffinose recovered.	Rotation	Reduction.	Rotation.	Reduction.	Rotation.	Reduction.
			cc.	*gm.*	*per cent*	*V°.*		*V°.*		*V°.*	
I. 2.1 kg	Feb. 22	4.21 gm. ro. intraperitoneally.	130	3.90	92.6						
	" 23		35	0							
	" 26	6.22 " " "	92	5.35	85.8						
	" 27		50	0							
	Mar. 1					+9.91	—	+9.88	—		
	" 5	25 cc. boiled ra. + 4.18 gm. ro. intravenously.	34	3.40	81.5						
	" 6		38	0							
	" 8					+9.90	—	+9.85	—		
	" 20					+9.85	—	+9.84	—	+9.82	—
II. 1.9 kg.	Feb. 22	3.82 gm. ro. intraperitoneally.	103	3.32	86.9						
	" 23		102	0							
	" 26	5.66 " " "	96	4.85	85.6						
	" 27		86	0							
	Mar. 3					+9.95	—	+9.90	—		

	Mar. 5	3.77 " " intravenously.	114	3.43	91.1						
	" 6		53	0							
	" 8					+9.90	–	+9.80	–		
	" 22					+9.80	–	+9.80	–	+9.80	–
	Mar. 12	25 cc. ra. + 4.36 gm. ro. intravenously.	167	3.97	91.1						
	" 13		105	0							
	" 20					+9.85	–	+9.85	–	+9.80	–
III. 2.3 kg.	" 21	35 cc. boiled ra. + 3.77 gm. ro. intravenously.	126	3.50	92.7						
	" 22		97	0							
	Apr. 2	35 cc. ra. + 3.70 gm. ro. intravenously.	223	3.22	87.0						
	" 3		103	0							

TABLE VI.

The Inversion of Raffinose in the Urine by Sulfuric Acid.

Urine sample.	Rabbit	I.	II.	III.	III.
	Date	Mar. 5.	Mar. 5.	Mar. 12.	Mar. 21.
Before inversion.	Rotation, $V°$	+15.30	+11.40	+9.00	+10.50
	Reduction	–	–	–	–
After partial inversion.	Rotation, $V°$	+ 7.50	+ 5.30	+4.61	+ 5.00
	Reduction	+++	+++	+++	+++
After total inversion.	Rotation, $V°$	+ 3.60	+ 2.30	+2.15	+ 2.10
	Reduction	+++	+++	+++	+++

to the circulation, are not eliminated quantitatively through the kidneys. Heilner ascribed the loss of part of the sucrose parenterally administered to the appearance of the corresponding enzyme in the serum (28). Abderhalden considered that sucrase which is called forth by parenteral injection of sucrose is probably mobilized from a special part of the body where this enzyme is physiologically produced; namely, from the mucous membrane of the small intestine (29). If these hypotheses are true, the extent of the recovery of sucrose injected parenterally may differ from those of other sugars, for which no corresponding enzymes exist in the organism. Inulase and raffinase have never been found in the animal body (20, 30, 31). Injecting 2.8 and 2.2 gm. of inulin intraperitoneally, Mendel and Mitchell recovered 2.2 and 1.43 gm. respectively of it in the urine (32). Being of a colloidal character, inulin may not be able to pass through the kidneys as easily as crystalloid sugars. From this viewpoint, the parenteral injection of raffinose, which is of a crystalloid character and finds no corresponding enzyme in the animal body, is of interest. Though Weinland and Abderhalden and Kapfberger claimed to find some special enzymes in the serum after parenteral administration of sucrose and lactose, they failed to obtain a comparable phenomenon after introduction of inulin (33, 34).

In a few experiments, therefore, we examined the utilization of raffinose parenterally administered and the raffinose-splitting power of the serum of the same animals. In Kuriyama's previous experiments, an injection of sucrase, followed a few minutes

later by sucrose injection, markedly increased the utilization of that sugar (27). Experiments of the same kind were performed with raffinose.

Methods.—Full grown rabbits were used. They were fed on oats and corn, greens being added from time to time. A 10 per cent raffinose solution, sterilized by boiling, was injected either into the peritoneal cavity or an ear vein. When raffinase and raffinose were injected successively, raffinase was always injected first, the interval between the two injections being 10 minutes. In control experiments, boiled enzyme solution was used. The serum examination for raffinase was performed as described on pp. 130 and 131. To activate the enzyme, 1 cc. of 0.075 N CH_3COOH was sometimes added to the raffinose-serum mixture. Raffinose in the urine was determined polarimetrically after removing the disturbing substances (25 cc. of urine + 10 cc. of saturated mercuric acetate solution).

In order to ascertain the nature of the dextrorotatory and non-reducing substance in the urine, the specimens were heated with sulfuric acid. For partial inversion (levulose + melibiose) the urine, mixed with sulfuric acid so that it contained 3.3 per cent sulfuric acid, was heated for 30 minutes at 75°C. For total inversion (levulose + glucose + galactose) the urine, acidified so that the mixture contained 1.2 per cent sulfuric acid, was heated for $6\frac{1}{2}$ hours at 100°C. The results are shown in Tables V and VI.

Of the raffinose, administered intravenously or intraperitoneally into rabbits in doses of 1.96 to 2.95 gm. per kilo of body weight, 88.4 per cent (as an average of six injections of raffinose alone) was recovered in the urine. The urine was always acid and contained neither reducing substance nor protein. After partial inversion of raffinose in the urine, the dextrorotation decreased to about one-half of the original degree; after total inversion to about one-fifth. These figures coincide with the properties of raffinose (35) and show that raffinose existed unchanged in the urine. When raffinase and raffinose were injected successively, no better utilization was called forth than when raffinose alone was injected. The difference between these experiments and the more favorable utilization of sucrose + sucrase (27) may be due to an insufficient amount of raffinase or the immediate disappearance of this enzyme from the circulation. The serum, taken from time to time, possessed no raffinose-splitting power, even in cases where the mixture was slightly acidified to facilitate the enzyme action.

TABLE VII.

Glycogen Formation in the Liver after Raffinose Feeding. The Fate of Raffinose in the Alimentary Tract. Sucrose Feeding for Control Experiments.

			I.	II.	III.	IV.	V.	VI.	VII.	VIII.	IX.	X
White rat	No.		I.	II.	III.	IV.	V.	VI.	VII.	VIII.	IX.	X
	Body weight, *gm.*		211	306	151	246	200	250	218	[illegible]	150	219
Loss of body weight after 3 day fast, *gm.*			26	47	28	35	22	48	43	38	30	37
Food after fasting			Raffinose, 5 gm.	Raffinose, 5 gm.	Raffinose, 2 gm.	Raffinose, 3 gm., casein, 3 gm.	Raffinose, 3 gm., casein, 3 gm.	None.	Casein, 5 gm.	Sucrose, 5 gm.	Sucrose, 2 gm.	Sucrose, 3 gm., casein, 3 gm.
Liver.	Weight, *gm.*		4.5	6.5	3.4	7.1	4.9	4.5	7.2	6.9	4.2	8.1
	Glycogen content.	*mg.*	Trace.	Trace.	Trace.	66.4	74.6	Trace.	202.4	399.6	216.1	464.7
		per cent.				0.94	1.52		2.81	5.79	5.15	5.74
Contents of stomach.	Appearance		Semi-fluid.	Soft.	Semi-fluid.	Soft.	Soft.	Empty (washed out).	Soft.	Soft.	Soft.	Soft.
	Reaction		Acid.	Acid.	Acid.	Acid.	Acid.	Neutral.	Acid.	Acid.	Acid.	Acid.
	Reduction	Before inversion.	–	–	±	–	–	–	–	–	–	+
		After inversion.	+	–	+	+++	++	–	–	–	–	+++

Contents of small intestine.	Appearance		Fluid.	Fluid.	Fluid.	Fluid.	Fluid.	Mucus (washed out).	Fluid.	Fluid.	Fluid.	Fluid.
	Reaction		Neutral.	Neutral.	Acid.	Acid.	Acid.	Neutral.	Acid.	Neutral.	Acid.	Acid.
	Reduction	Before inversion.	–	–	–	–	–	–	–	–	–	–
		After inversion.	+++	++	++	+++	++	–	–	–	–	–
Contents of large intestine.	Appearance		Fluid.	Fluid.	Fluid.	Fluid.	Fluid.	Mucus (washed out).	Soft.	Soft.	Semifluid.	Soft.
	Reaction		Neutral.	Neutral.	Acid.	Acid.	Acid.	Neutral.	Neutral.	Neutral.	Neutral.	Acid.
	Reduction	Before inversion.	–	+++	+++	+	+++	–	–	–	–	–
		After inversion.	+++	+++	+++	++	+++	–	–	–	–	–
Excreta (feces + urine).	Appearance		Watery feces.	Watery feces.	No feces.	Watery feces.	No feces.	No feces.	Hard feces.	Hard feces.	No feces.	Hard feces.
	Reaction		Alkaline.	Neutral.	Neutral.	Acid.	Acid.	Acid.	Acid.	Acid.	Acid.	Acid.
	Reduction	Before inversion.	+	++	–	+	–	–	–	++	+	±
		After inversion.	+++	+++	+++	++	+++	–	–	++	++	±

Glycogen Formation in the Liver after Raffinose Feeding; the Fate of Raffinose in the Alimentary Tract.

Judging from Külz's and Sandmeyer's reports (10, 12), the utilization of raffinose in the alimentary tract seems to be rather difficult. Halász's experiments suggest that part of it can be inverted in the rectum (14). Some experiments in this direction were performed on white rats.

Methods.—Full grown white rats fasted for 72 hours. This period was so arranged that it ended at 9 p.m. A certain amount of selected food was then left in the cage over night. At 9 the next morning the animal was decapitated and the glycogen content of the liver was determined by Pflüger's method. The glycogen was hydrolyzed with hydrochloric acid and the reducing sugar produced was determined by Allihn's gravimetric method. The contents of the stomach and small and large intestine were collected separately. Each portion was diluted with water to about 10 cc. The filtered contents were tested for reducing power both before and after hydrolysis with 3.3 per cent sulfuric acid for 30 minutes at 75°C. The urine and feces were collected together. They were unavoidably contaminated with particles of the food. The food was given in the form of paste. As raffinose causes severe diarrhea, casein was mixed with it in some experiments, in the hope of decreasing the purgative action of the raffinose. For control experiments, sucrose and casein or either sucrose or casein alone were given.

From Table VII it will be seen that after raffinose feeding, glycogen was not formed in the liver to any noticeable extent. Though raffinose was present in the stomach and small intestine, no reducing substance was found. It may be that the cleavage products of raffinose were very easily absorbed and could not be detected in the stomach and small intestine. Judging from the absence of glycogen formation in the liver and the failure to find raffinase in the intestinal mucous membrane of vertebrate animals, it is very likely that raffinose was not inverted, at least to any noteworthy amount, in the stomach and small intestine. In the large intestine, however, some reducing substances were found, probably owing to the bacterial action. This point will be discussed later.

Why did not the reducing substances, produced in the large intestine, become a source of glycogen in the liver? Was the amount of the cleavage products of raffinose insufficient or were the monosaccharides further destroyed in the intestinal canal,

without entering into the circulation? Studying the fate of dextrose in the large intestine and in a feces-dextrose mixture *in vitro*, Bingel concluded that the sugar was absorbed very slowly in the large intestine and the amount of the sugar destroyed by bacteria was as great as that absorbed (36). Introducing a large amount of sugars (mono-, di-, and trisaccharides) into the large intestine, Halász found that a noteworthy amount of the sugar was absorbed in 5 to 6 hours, the amount destroyed by bacteria being rather negligible. For the absence of the sugar in the urine in his experiments, the slow absorption in the large intestine was considered to be one reason (14). In our experiments, diarrhea caused by raffinose seems also to have been a factor explaining absence of glycogen from the liver.

In control experiments with sucrose feeding, the food was easily inverted and became a source of glycogen in the liver. When casein was added to raffinose, some glycogen formation was observed. In these cases, raffinose may not have passed so quickly through the alimentary tract as when this sugar was given alone, and consequently may by chance have been inverted. According to Bendix' and Stookey's experiments, casein alone can become a source of some glycogen in the liver (37, 38). Rat VII in our experiments seems to confirm their results. It is therefore probable that the liver glycogen obtained after raffinose-casein feeding is at least for the most part due to casein itself.

The behavior of raffinose in the alimentary tract is somewhat similar to that of inulin. The glycogen formation in the liver after inulin feeding to fasting rabbits is slight or uncertain (39, 40). After inulin feeding, Miura found a reducing substance in the stomach and large intestine, also sometimes in the small intestine.

The Fate of Raffinose Administered into Intestinal Loops of Dogs.

In the preceding rat experiments raffinose was inverted in the large gut, but not in the small intestine. To examine this point further, we studied the fate of the sugar, introduced into intestinal loops of dogs.

Methods.—Full grown dogs fasted for 48 hours before operation. The urine contained neither protein nor sugar. As anesthetics, urethane

TABLE VIII.

The Fate of Raffinose, Introduced into Intestinal Loops of Dogs.

			I. ♀	II. ♀	III. ♂
Dog.		No. and sex	I. ♀	II. ♀	III. ♂
		Body weight, *kg*	9.9	11.4	9.2
Anesthetic.			Urethane (6.0 gm.) + ether.	Urethane (6.4 gm.) + ether.	Urethane (4.6 gm.) + ether.
Upper loop of small intestine.	Length of loop, *cm*		62	28	45
	Amount of raffinose introduced, *gm*		2.00	2.00	2.00
	Irrigation fluid.	Volume, *cc*	255	290	245
		Reaction	Neutral.	Neutral.	Neutral.
		Reduction	—	—	—
		Raffinose recovered. *gm*	1.95	1.89	1.94
		Raffinose recovered. *per cent.*	97.5	94.5	97.1
		Osazone	—	—	—
Lower loop of small intestine.	Length of loop, *cm*		80	30	53
	Amount of raffinose introduced, *gm*		2.00	2.00	2.00
	Irrigation fluid.	Volume, *cc*	208	249	281
		Reaction	Neutral.	Neutral.	Neutral.
		Reduction	—	—	—
		Raffinose recovered. *gm*	1.69	1.13	1.89
		Raffinose recovered. *per cent.*	84.5	56.5	94.5
		Osazone	—	—	—

Loop of large intestine.	Length of loop, *cm*		13	15	12
	Amount of raffinose introduced, *gm*		2.00	2.00	2.00
	Irrigation fluid.	Volume, *cc*	251	252	278
		Reaction	Acid.	Acid.	Acid.
		*Reducing substance, *gm*	0.41	0.57	0.88
		**Raffinose recovered. *gm*	1.41	1.22	0.92
		**Raffinose recovered. *per cent.*	70.5	61.0	46.0
		Osazone	+	+	+
Urine.	Volume, *cc*		29	62	35
	Reaction		Acid.	Acid.	Acid.
	Reducing substance, calculated as glucose, *gm*		2.20 from rotation 2.22 " reduction	0	0
	Raffinose, calculated from rotation, *gm*		0	0.18	0.03
	Osazone		+	—	—

* The amount of reducing substance was calculated to be a mixture of equal amounts of glucose, levulose, and galactose.

** The amount of raffinose was calculated from the difference between rotation and reduction.

(applied subcutaneously) and ether were used. Two loops were established on the small intestine: the upper end of the first loop was at the beginning of the duodenum, leaving the opening of the bile and pancreatic duct within the loop; the lower end of the second loop of the small intestine was just above the ileocecal valve. The loop on the large intestine began a few centimeters from the cecum. Glass cannulas were inserted into both ends of the loops. 20 cc. of a 10 per cent raffinose solution were introduced from the upper end of each loop and the intestine was gently massaged, so that the raffinose solution was distributed through the whole length of the loops. Every precaution was taken not to damage the intestinal mucous membrane and the blood vessels providing the alimentary tract. After closing the abdominal cavity, the animal was kept warm and under a slight anesthesia for 2 hours. The contents of the loops were then washed out with a warmed physiological saline solution. The fluid discharging from the lower end of the loop was mixed thoroughly with toluene. After killing the animal by bleeding, all the loops were again washed out. The remaining parts of the intestinal canal (the middle part of the small intestine and the beginning and end parts of the large intestine) were also washed out. The urine was collected from the bladder. The proteins in the irrigation fluid were removed by colloidal iron solution (25 cc. of fluid + 5 or 10 cc. of colloidal iron solution). In the water clear filtrate the reducing substance was determined by Allihn's gravimetric method. The fluids, obtained by washing the remaining parts of the intestine and the second washing of the loops, showed neither reducing power nor rotation. Before determining the rotation of the urine, it was clarified with mercuric acetate.

As Table VIII clearly shows, raffinose was very slowly absorbed by the small intestine, especially by the upper part. No evidence of its inversion was observed. In order to determine the nature of the dextrorotatory substance, obtained from the small intestine, the protein-free irrigation fluid was heated with sulfuric acid as described previously. After partial inversion the rotation decreased to nearly half of the original degree, and after total inversion to nearly one-fifth (Table IX). This corresponds to the properties of raffinose (35).

The slow absorption of raffinose in the small intestine in our experiments is noticeable. There have been many investigations of the absorption of various kinds of sugar from the small intestine (41, 42, 13, 43). For full grown dogs Röhmann and Nagano reported that among the disaccharides sucrose was absorbed the most quickly, lactose the most slowly. No evidence of the inversion of lactose was observed, while sucrose and maltose were partly inverted before their absorption (42). Comparing the

TABLE IX.

The Inversion of Raffinose, Recovered from the Intestinal Loops, by Sulfuric Acid.

Sample.	Dog	I.		II.		III.	
	Loop of small intestine.	Upper loop.	Lower loop.	Upper loop.	Lower loop.	Upper loop.	Lower loop.
Before inversion.	Rotation, $V°$	+3.85	+4.10	+3.22	+2.28	+4.00	+3.40
	Reduction	–	–	–	–	–	–
After partial inversion.	Rotation, $V°$	+1.70	+1.80	+1.50	+1.19	+2.04	+1.69
	Reduction	+++	+++	+++	+++	+++	+++
After total inversion.	Rotation, $V°$	+0.80	+0.80				
	Reduction	+++	+++				

rapidity of absorption of various kinds of sugar (20 cc. of 25 per cent solution were introduced) in a loop 1 meter long of the small intestine of rabbits, Hédon found that raffinose disappeared most slowly. 10.2 per cent of the amount introduced was absorbed in 2 hours. The rapidity of absorption increased gradually in the following order: lactose, maltose, sucrose, levulose, etc. (13).

The irrigation fluid from the loop of the large intestine contained a noteworthy amount of reducing substances. In the osazone test, yellow crystals appeared while heating, and increased markedly after cooling. Microscopically, most of the crystals had the appearance of phenylglucosazone but some the shape of phenyllactosazone. This suggests the production of a small amount of melibiose (35) together with other cleavage products. As the exact nature of the reducing substances was not clear, the amount was calculated to be a mixture of equal portions of glucose, levulose, and galactose. The raffinose was calculated from the difference between rotation and reduction.

In the urine of Dog I, a large amount of glucose was found, contrary to the other two experiments. The amount of reducing substances produced in the large intestine was not enough to explain this glycosuria. The operation and anesthesia may have been the cause. In the urines of Dogs II and III, a small amount of a dextrorotatory and non-reducing substance was found. After acid hydrolysis, it showed reducing power. This substance was calculated to be raffinose. The fact that raffinose can pass

unchanged through the intestinal wall and reappear in the urine was also demonstrated by other investigators (12, 44).

The Presence of Raffinase in the Feces.

The inversion of raffinose in the large intestine seems to be due to bacteria. In reality, numerous kinds of bacteria have the power to invert raffinose. Investigating the properties of seventy-seven strains of colon bacilli, isolated from polluted water, feces, urine, and the animal body, Kligler found that forty-one strains of them had the ability to attack raffinose (45). In the study of 350 strains of streptococci, isolated from human, equine, and bovine feces, Fuller and Armstrong found that in human feces none, in horse feces 12 per cent, and in cow feces 73 per cent of the strains examined attacked raffinose (46). It is probable that such bacteria act favorably to the host, by decomposing raffinose into utilizable forms. A comparable phenomenon has also been reported for cellulose and intestinal bacteria (47). Though raffinose is usually first decomposed into levulose and melibiose, it can also be inverted into galactose and sucrose (48). It is yet unknown whether the latter mode of inversion can also occur in the intestinal canal.

When the fluids, obtained from the loops of the large intestine and mixed thoroughly with plenty of toluene, were kept for 48 hours at room temperature, their rotatory power decreased markedly. This was not the case, however, with the fluids from the small intestine (Table X).

TABLE X.

The Examination of Rotation of the Irrigation Fluids Both Immediately and after Keeping 48 Hours at Room Temperature with Toluene Addition.

Fluid obtained from	Examination.	Dog II.	Dog III.
		$V°$.	$V°$.
Upper loop of small intestine.	Immediately	+3.28	+4.00
	After 48 hrs.	+3.28	+4.00
Lower loop of small intestine	Immediately	+2.28	+3.40
	After 48 hrs.	+2.30	+3.38
Loop of large intestine	Immediately	+2.30	+1.70
	After 48 hrs.	+0.40	+0.60

Raffinose or its cleavage products in the irrigation fluid of the large intestine, therefore, must have been attacked either by active bacteria or isolated enzyme. Though Ury stated that toluene addition was enough to eliminate the bacterial action in diastase determination in the feces (49), our bacteriological examination showed that our materials, obtained from the large intestine and mixed thoroughly with toluene, were not sterile.

To make a sterile fecal extract, therefore, fresh dog and rabbit feces, obtained from an animal room, were ground with about ten volumes of water and mixed with both toluene and chloroform. 7 to 8 hours later the mixture was filtered through cloth. The slightly acid filtrate was used for the digestion test. The procedure was the same as described previously. Cultivating from these samples, no bacterial growth was observed on agar-agar media in 48 hours. For control, the feces extract was previously boiled or mixed with mercuric chloride.

The results with these extracts of the feces of dogs and rabbits indicate a small amount of raffinase, probably originating from bacteria. The feces extract can be more properly sterilized with a porcelain filter (50, 51). Our investigation of raffinase in the feces is not yet concluded.

SUMMARY.

A hydrogen ion concentration of pH 3.8–5.4 is the most favorable for the activity of raffinase (from yeast).

Saliva (human), bile (rabbit), pancreas (dog), liver (dog and rabbit), and mucous membrane of small and large intestine (dog and rabbit) do not contain raffinase. The gastric juice may invert raffinose under suitable conditions.

Blood serum (rabbit) is not a favorable medium for the activity of yeast raffinase; yet a sufficient quantity of the enzyme can exert activity. When yeast raffinase is injected intravenously into rabbits, the activity of the enzyme can be maintained in the serum for a short time only.

When raffinose was injected parenterally into rabbits in doses of about 2 to 3 gm. per kilo of body weight, 88 per cent of the amount administered was recovered in the urine. The serum of the same animals failed to show raffinose-splitting power.

When raffinase and raffinose were injected successively into

the circulation, no better utilization of the sugar was called forth than when raffinose alone was injected.

No noteworthy glycogen formation in the liver was found after feeding raffinose to fasting white rats. The sugar was scarcely inverted in the stomach and small intestine. It was, however, changed in the large intestine.

When raffinose was administered directly into loops of the small intestine of dogs, most of it was recovered after 2 hours without evidence of its inversion. In a loop of the large intestine, however, raffinose was easily inverted. This was probably due to bacteria. Part of raffinose can pass unchanged through the intestinal wall and reappear in the urine. In our experiments, the animals (rats and dogs) were previously fasted for a few days. This might have been unfavorable for the growth of intestinal bacteria, especially for raffinose-attacking bacteria. Under ordinary conditions, therefore, the inversion of raffinose may occur more extensively and perhaps even in the small intestine.

The sterilized feces of dogs and rabbits seems to contain a small amount of raffinase, probably of bacterial origin.

Raffinose is devoid of food value until after its inversion. It may be that raffinose-digesting bacteria occur more frequently in the large intestine of species which consume foods containing raffinose and thus render the physiological utilization more probable for them.

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THE RÔLE OF VITAMINES IN THE DIET.*

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The feeding experiments first made by Hopkins[1] have led to widespread recognition of the importance of small quantities of hitherto unidentified substances—in addition to the protein, carbohydrate, salts, and fat—as essential components of a ration adequate for prolonged maintenance or growth. The development of our own views on this subject has been alluded to in a recent publication.[2] Röhmann,[3] however, has taken vigorous exception to the vitamine hypothesis. He asserts that "accessory foodstuffs are not necessary for the continued maintenance of fully grown animals,"[4] and believes that if the long familiar nutrients are suitable in quality and quantity, nothing further is essential in the ration. Thus he says: "The assumption that some unknown substances are indispensable for growth is a convenient device for explaining experiments that result in failure—a device that becomes superfluous as soon as the experiment succeeds."[5]

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

[1] Hopkins, F. G., *J. Physiol.*, 1912, xliv, 425.

[2] Osborne, T. B., and Mendel, L. B., *Biochem. J.*, 1916, x, 534.

[3] Röhmann, F., Ueber künstliche Ernährung und Vitamine, Berlin, 1916.

[4] "'Akzessorische' Nahrungsstoffe sind mindestens zur dauernden Erhaltung ausgewachsener Tiere nicht notwendig" (p. 142).

[5] "Die Annahme von irgendwelchen unbekannten Stoffen, die für das Wachstum unentbehrlich sind, ist ein bequemes Mittel, um die fehlgeschlagenen Versuche zu erklären, das überflüssig wird, sobald der Versuch gelingt" (p. 42).

As evidence against the indispensability of vitamines Röhmann cites a new series of experiments on the growth and maintenance of white mice fed with artificial mixtures of food supposedly free from these so called accessory substances. It will be unnecessary to review the successive steps in his extensive investigations, because yeast was used to impart a suitable texture to many of the food mixtures earlier employed by him. The efficiency of yeast as a source of vitamine, to which we can testify from our own experience, has long been recognized. To meet this criticism Röhmann used, in his latest experiments, starch digested by "purified" diastase, and leavened the mixtures with baking powder. We shall therefore concentrate attention upon the crucial experiments in his newer series in which the diets had the following composition:

*1. Hühnereiweissnahrung.**	*gm.*	*2. Kasein-Vitaminfreie Nahrung.***	*gm.*
Egg white	5	"Kalzose"	22
Egg white iron	2	Casein-iron	2
Potato starch (predigested)	20	Potato starch (predigested)	20
Potato starch (raw)	25	Potato starch (raw)	25
Wheat starch	90	Wheat starch	90
Dextrose	5	Dextrose	20
Margarine	10	Lard	24
Salts	3	Salts	6
Baking powder	5	Baking powder	5

* Pages 27–28. ** Page 43.

Röhmann states that with Diet 1 five young mice having an average initial weight of 6 gm. were successfully reared. In comparison with most data on the feeding of growing animals the protein (4.4 to 10 per cent) was surprisingly low. Addition of casein gave still more successful results, for a third generation was obtained. It should be noted that the egg white used in these experiments was not purified, but "albumen ovi siccum dried in vacuum," of which nothing is known respecting its effect on growth. The use of diastase is also open to criticism, since McCollum[6] has shown that extracts of some of the cereal grains possess decided growth-promoting properties.

In subsequent experiments with Diet 2 Röhmann met the possible objection to the presence of vitamine by the statement that "the food stuffs were either treated with alcohol *without warming*, or were heated 3 hours at 120–150°" (p. 42).

The *"Kalzose"* used was a commercial preparation of calcium-casein admittedly still containing some milk sugar, and doubtless other products present in milk. In this series 0.2 gm. of "Merck's purified diastase," washed with alcohol, also was used to digest 20 gm. of starch.

[6] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 181, 231.

With Diet 2 Röhmann states that he has succeeded in rearing young mice and subsequently maintaining them. His protocols, however, show that in some cases some of the mice were consumed by the others, and success was frequently induced not only by added alcoholic extracts of yeast or by small quantities of milk, but particularly with "*Filtrateiweiss*," a product from milk "principally composed of proteins which remain in solution after the precipitation of the casein" (p. 42). According to his account of the method of preparation this product may have contained some or all of the other constituents of milk among which are those proved to be especially efficient in promoting growth.

Presumably relying on the earlier statements regarding the loss of antiscorbutic power in various natural substances which have been heated, Röhmann considered the application of heat or extraction with cold alcohol to be sufficient to exclude the presence of vitamines. The effect of heat has been discussed by Funk.[7] The destruction by heat of both the fat-soluble and water-soluble vitamines present in milk is improbable.[8] Furthermore, considerable difference of opinion regarding the relation of heated milk to infantile scorbutus still exists.

Alcohol was doubtless used by Röhmann in view of the statement of various investigators since Hopkins that this substance is a solvent for the vitamine of yeast, milk, wheat embryo, etc. Whether Röhmann used *absolute* alcohol or alcohol containing more or less water to remove vitamines is not apparent from his published descriptions. Our own experience discussed below has shown that *absolute* alcohol is by no means an adequate solvent for the effective nutrition-promoting accessory substances in yeast. We have not yet learned how much water the alcohol must contain in order to extract these substances. It is doubtful therefore whether either of the methods selected by Röhmann can be depended upon to exclude all traces of vitamines.

The thesis that a successful, *i.e.*, positive, experiment in nutrition is far more significant than a negative one is doubtless valid. On the other hand, in dealing with substances which, like the alleged vitamines, are potent in surprisingly small amounts, the burden of proof with respect to the complete absence of effective substances so widely distributed among the natural foodstuffs falls on those who deny the need for them. Without the use of some water-soluble accessory substances such as has been dem-

[7] Funk, C., *Ergebn. Physiol.*, 1913, xiii, 125.

[8] Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 381. McCollum and Davis, *ibid.*, 1915, xxiii, 247.

onstrated to be present in milk,[1] "protein-free milk,"[9] yeast,[10] the extract of embryos of certain seeds,[6] animal tissue extracts,[11] or doubtless in nearly all the commonly used animal or vegetable foods in their natural state, we, in common with many other investigators, have failed to induce growth or even maintenance in such a large proportion of our trials that the importance of the vitamine hypothesis has been forced upon us. Nevertheless, occasionally, though very infrequently, an animal has for a time grown well or been maintained for exceptionally long periods on a ration of isolated food substances supposedly free from all but traces of water-soluble vitamine, upon which an overwhelming majority of the same species promptly fails to thrive.[12] For example, Rat 3030♂ on a diet of lactalbumin, "artificial protein-free milk," starch, lard, and butter fat, grew from 286 gm. to 372 gm. and showed no signs of nutritive failure after 392 days. This food contained purified lactose. It may still have contained a trace of vitamine, though we regard this as an unlikely explanation of the outcome of the preceding experiment, in view of our numerous failures with artificial foods containing lactose from the same stock. From these very infrequent successes it would seem as if such a diet were adequate for maintenance provided that an animal can be induced to consume enough of it. One of the immediate effects of the addition of "protein-free milk," small quantities of yeast, etc., to such a ration is an improved appetite attended by increase of weight. This is well exemplified in Chart I in which the food intakes on the different diets are arranged for comparison.

It is now generally believed that if a young rat is fed on a diet consisting of purified protein, carbohydrate, lard, a suitable mixture of inorganic salts, along with some water-soluble vitamine,

[9] Osborne and Mendel, *Carnegie Institution of Washington, Publication No. 156*, pt. ii, 1911; *Z. physiol. Chem.*, 1912, lxxx, 356; *J. Biol. Chem.*, 1913, xv, 311.

[10] Funk, *J. Biol. Chem.*, 1916, xxvii, 1. Funk, C., and Macallum, A. B., *ibid.*, 1915, xxiii, 413; 1916, xxvii, 51. Hopkins, *J. Physiol.*, 1912, xliv, 425.

[11] Eddy, W. H., *J. Biol. Chem.*, 1916, xxvii, 113.

[12] Osborne and Mendel, *J. Biol. Chem.*, 1912–13, xiii, 233; 1913, xv 311. McCollum and Davis, *ibid.*, 1913, xv, 167.

it will fail to complete its growth.[13] Replacing a part of the lard by butter fat,[14] egg yolk fat,[15] beef fat,[16] or cod liver oil[17] renders the ration adequate for promoting normal growth and reproduction provided that the protein of the ration is also suitable. Experience has therefore demonstrated that adequate dietaries require at least *two* formerly unappreciated components.

Hopkins,[1] who first thus added yeast to a diet of purified food stuffs, reported that very small quantities of a protein-free alcoholic extract or even of the ether-soluble fraction of the alcoholic extract of yeast markedly accelerated the growth of rats. Hopkins writes:

"There is some indication from my experiments that the optimum supply of the substances which induce growth is soon reached; but any attempt to ascertain the nature of their action by noting the relation between their concentration and their effects would call for extensive experimentation which it would seem better to leave until definite substances have been isolated" (p. 440).

Recently Funk and Macallum[10] have shown that yeast added to artificial diets for growing rats accelerates their growth. Funk[10] states:

"Our experiments show that the quantity of vitamines necessary for stimulating growth in rats is by no means small. If yeast is added to the diet to the extent of 1 per cent the rats grow for a short time, after which they begin to decline. Experiments which are not recorded in this paper have shown that at least 3 per cent of yeast is necessary to insure a satisfactory growth in rats. Still further experiments have shown that yeast has more effect in promoting growth than an addition of a few cc. of milk, as used by Hopkins. Yeast can be regarded as a complete food by itself. It was therefore necessary to ascertain whether the good results obtained with this addition are not merely due to a correction of the nutritive value

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[13] Osborne and Mendel, *Z. physiol. Chem.*, 1912, lxxx, 356; *J. Biol. Chem.*, 1913, xv, 311. McCollum and Davis, *ibid.*, 1913, xiv, p. xl; 1913, xv, 167. Hopkins, *J. Physiol.*, 1912, xliv, 425.

[14] Osborne and Mendel, *J. Biol. Chem.*, 1913–14, xvi, 423; McCollum and Davis, *ibid.*, 1913, xv, 167.

[15] MacArthur, C. G., and Luckett, C. L., *J. Biol. Chem.*, 1915, xx, 161. Osborne and Mendel, *ibid.*, 1914, xvii, 401; 1915, xx, 379. McCollum and Davis, *ibid.*, 1913, xv, 167.

[16] Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 379.

[17] Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 401.

of the protein used (in this case, casein) or to the presence in it of nucleic acid. Consequently a diet was prepared in which the total casein nitrogen was substituted by yeast nitrogen. The results obtained were not as satisfactory as when yeast was used in smaller amounts for its vitamine content only, and not for nutritive value."

Yeast has frequently been used to cure polyneuritis induced in birds by a diet of polished rice. Whether or not the antineuritic component is identical with the growth-promoting one is a question which as yet has received no definite answer, although Funk and Macallum think that their results "indicate that the growth-promoting substance is analogous to and possibly identical with the beri-beri vitamine" but that "considerably larger quantities of vitamines are necessary for stimulating growth than for curing beri-beri."[18]

Seidell[19] reports that Lloyd's reagent completely removes from autolyzed brewers' yeast a substance capable of preventing polyneuritis in pigeons fed on polished rice.

On the other hand Gibson and Concepción[20] found that puppies and pigs even while growing develop symptoms of peripheral nerve degeneration when milk, whether fresh or heated, forms their sole diet.

Our early experiments with "artificial protein-free milk"[21] indicated its marked inferiority to our "natural protein-free milk" when fed to growing rats, although the product contained like proportions of everything known to be present in the natural product. The fact that in our earlier experiments rats occasionally grew well on the "artificial" diet led us at first to suspect that some essential inorganic element was present as an impurity in the chemicals which we then used. Our later experience has, however, inclined us to the conclusion that the inorganic content of all these salt mixtures was probably suitable, but that the dietary deficiency lay in the lack of some essential undetermined organic food factor.

To render our "artificial" food mixtures as efficient for pro-

[18] Funk and Macallum, *J. Biol. Chem.*, 1916, xxvii, 63.

[19] Seidell, A., *Public Health Report No. 325*, 1916.

[20] Gibson, R. B., and Concepción, I., *Philippine J. Sc., B*, 1916, xi, 119.

[21] Osborne and Mendel, *J. Biol. Chem.*, 1913, xv, 311.

moting growth as the foods containing our "natural protein-free milk," we have recently fed small quantities of dried brewers' yeast either separately or incorporated in the artificial food mixtures. On a ration of purified casein, "artificial protein-free milk," starch, lard, butter fat, and 1.5 per cent of dried yeast, rats of both sexes have grown from about 50 gm. body weight to maturity, and have even produced young. This is a smaller proportion of yeast than Funk considered necessary for normal growth. Adult rats have been maintained for more than 300 days. For some as yet unknown reason the majority of the rats grew normally when the protein used was casein, whereas they have usually failed when it was edestin, and almost invariably when lactalbumin, cotton seed globulin, cotton seed proteins, or squash seed globulin was fed. This result surprised us because all of these proteins had earlier led to normal growth when used in rations containing natural "protein-free milk." The failure to grow on the "artificial protein-free milk"-yeast foods was especially unexpected in the case of lactalbumin (Chart II); for our former experience had demonstrated that even exceptionally small proportions of this protein promoted normal growth.[22]

The fact that in all of our numerous experiments with this lactalbumin-"artificial protein-free milk" diet young rats failed to grow with an addition of 1.5 per cent of yeast, and all soon died unless a change was made in the diet, whereas mature rats were maintained over very long periods, suggests that the unknown nitrogenous constituents of milk[23] may possibly supplement some hitherto unrecognized deficiency in this protein. Attempts to find an explanation for this unexpected result have not yet given us a clue.

McCollum and Davis[24] consider that the nitrogen of "protein-free milk" has essentially the same nutritive value as that of the milk proteins; but this assumption does not help to explain our results obtained with lactalbumin, for in this case we are confronted by an apparent deficiency in the chemical make-up of this protein which may possibly be supplemented by the unknown

[22] Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 351; 1916, xxvi, 1.

[23] Our "protein-free milk" has been found to contain, on an average, 0.68 per cent N.

[24] McCollum and Davis, *J. Biol. Chem.*, 1915, xx, 641.

constituents of milk. More precise knowledge of these latter, as well as of the products of hydrolysis of lactalbumin, is needed before definite conclusions can be reached. In contrast to casein (and perhaps edestin) some of the other proteins which we have fed may need a supplement which is not found in yeast, yet is present in the so called "protein-free milk." Whatever the nutritive value of the unknown constituents of milk may be, our experience manifestly leads to the conclusion that the supplementary value of "protein-free milk" in the diet is, as a general rule, decidedly greater than that of yeast. Indeed the value of the latter would perhaps not readily have been discovered if other proteins than casein had formed the basis for the food mixtures with which the earlier experiments were conducted.

The objection may be raised that since some rats grow on the "artificial" diets alone, those animals which have done well on the yeast diets might have grown equally well without it. The improbability of this assumption is shown by the fact that whereas the great majority of the rats without yeast failed to thrive after a comparatively short period, nearly all of those receiving yeast with an appropriate protein as already indicated continued to grow normally for a long time. Moreover, the removal of the yeast was almost invariably followed by immediate cessation of growth and ultimate decline which could be promptly checked and converted into rapid recovery by the addition of a small amount of yeast.

The numerous failures of growth in the experiments without yeast or other intentionally added sources of vitamines demonstrate that the quantities present in our other food ingredients must be far too small, at the best, to furnish enough of the growth-promoting material to satisfy the requirements of the majority of the animals tested. This experience does not support the suggestion made by some investigators,[25] that such ingredients of our foods as casein and lactose, prepared from milk, and used in many of our food mixtures, are the carriers of a sufficient quantity of vitamines to vitiate experiments designed to test the effect of specially added accessory materials of this class.

Our experiments indicate that the rapidity of growth is related

[25] McCollum and Davis, *J. Biol. Chem.*, 1915, xxiii, 181 and 231. Funk and Macallum, *ibid.*, 1916, xxvii, 62.

to the quantity of yeast fed. While there is a considerable variation among the individual rats with respect to the quantity of yeast needed, in general we have found 1.5 to 2 per cent of yeast in the food sufficient for promoting normal growth. Some rats have grown well with only 0.25 to 1 per cent of yeast in the diet, but almost invariably in these cases an increase in the amount of yeast given was followed by an increase in the rate of growth (Chart III). It might be assumed that when the rats grew on the smaller quantities of yeast their total food intake was greatly increased, so that the actual absolute intake of yeast was not very different from that of those animals receiving relatively larger percentages of yeast in their ration. A study of the food intakes of these rats, however, shows that this is not the case. The food consumption of the rats on the smaller quantities of yeast was less than that of those on the larger quantities, because their growth was slower and consequently they needed less food; and the change from a small quantity of yeast to a larger one was followed by growth with a resultant increase in the food intake.

How does the yeast exert its beneficial effect? Does it merely add something which renders the food more palatable and so stimulates the animal to eat more liberally of it? Or does it exert some favorable influence upon the metabolism of the rat, and thus improve its general condition so that more food is consumed? Satisfactory growth is associated with liberal eating; but whether the animal eats because it grows or grows because it eats is a difficult question to settle. Hopkins[1] believes that "any effect of the addendum upon appetite must have been secondary to a more direct effect upon growth-processes;" and our experience leads us to the same belief, especially in those experiments in which the yeast was fed separately. In these cases the yeast could not have affected the inherent palatability of the ration, and the fact that the rats immediately increased their consumption of the "artificial" food mixture points toward an improvement in the general condition of the animals which led them to raise their level of food consumption to keep pace with their more rapid growth and consequent need of more food. This experience fully confirms Hopkins' results with feeding separately small quantities of milk.[26]

[26] Cf. Hopkins for a full discussion (p. 441) of the relation of food intake to growth, which is confirmed by our own experience.

An attempt was made to concentrate the effective substance in the yeast by fractionation with alcohol. Moist yeast obtained from a brewery was filtered and the residue subjected to hydraulic pressure. The press cake thus obtained was heated to boiling in a large volume of distilled water made slightly acid with acetic acid, and filtered. The filtrate was evaporated and the resulting pasty mass thoroughly mixed with *absolute* alcohol and evaporated. After repeated evaporations with frequent additions of absolute alcohol the almost brittle mass was ground with absolute alcohol and centrifuged. The alcoholic solution, containing solids equal to 2.1 per cent of the dry yeast, was concentrated in a vacuum at about 65°C. The portion insoluble in absolute alcohol was pressed in the hydraulic press and dried in a vacuum over sulfuric acid. This was equal to 16.2 per cent of the dry yeast. Each of these fractions was incorporated with our "artificial protein-free milk" and fed in suitable rations to rats. The fraction soluble in absolute alcohol exerted no beneficial influence on their growth, whereas the addition of the residue soluble in water but insoluble in absolute alcohol in nearly every case led to a marked increase in their rate of growth. Here again the need of a sufficient quantity of the water-soluble fraction was demonstrated; for the use of 0.5 per cent of the yeast residue in the food was followed by a failure to make normal growth, whereas with 2 per cent of it in the food resumption of growth took place (Chart IV). That the above method of preparation has not tended to concentrate the effective substance in the yeast is shown by the fact that it required just as much of this residue, representing 16.2 per cent of the dry yeast, as it does of the whole yeast, to induce normal growth when fed with the "artificial protein-free milk" foods.

The experiments just described confirm the presence in yeast of something comparable with the so called water-soluble vitamine. They offer no evidence regarding the presence or absence of the fat-soluble one, since a liberal supply of butter fat was used in all of the food mixtures. Funk and Macallum found that diets which contained yeast and butter showed only a slight superiority over those which contained yeast and lard. Our own experiments in which lard was the only fat component indicate that yeast contains only a very small amount, if any, of the fat-

soluble factor. The lack of the butter fat may help to explain Funk and Macallum's failures with the phosphotungstic acid fractions of yeast;[18] for unless all the other necessary factors of the diet are adequately supplied, the presence or absence of the water-soluble vitamine cannot be demonstrated.

Despite the success which has attended the use of yeast as an adjuvant to otherwise inadequate food mixtures, notably in the case where casein or edestin furnished the bulk of the protein, such yeast-containing "artificial" food mixtures have not yet demonstrated a nutrient efficiency equivalent to that manifested through the use of "protein-free milk" or certain other naturally occurring food products like cotton seed meal. The refusal of some rats to eat an adequate amount of the yeast-containing foods has proved a stumbling block to exact comparisons. Although some of the animals brought up on the yeast-containing foods have given birth to young, thus far none of the latter has been reared.

The charts follow.

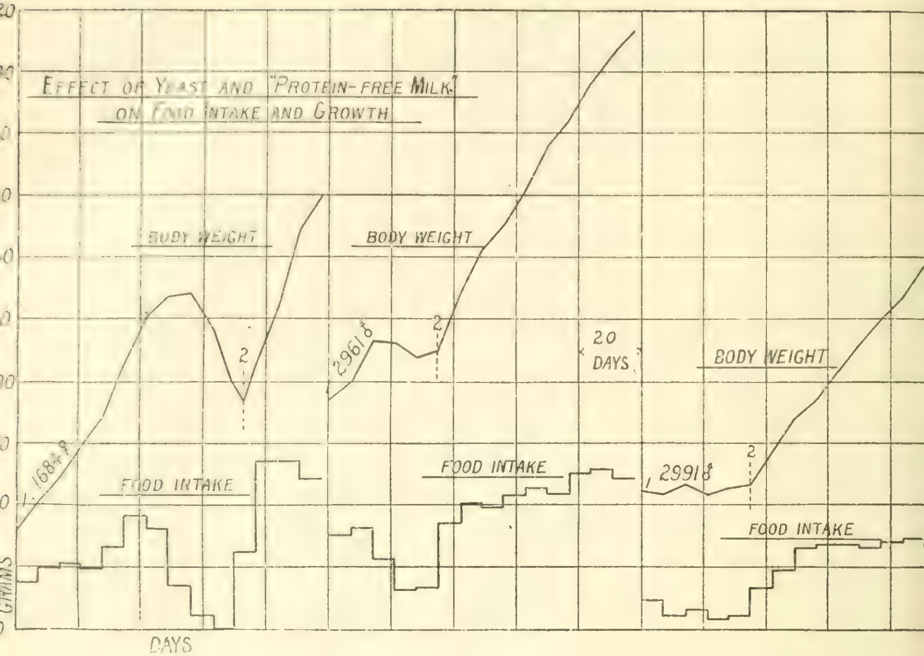

CHART I. Illustrative graphs showing the effects of small additions of yeast and of "protein-free milk" respectively on the rate of growth and the intake of food (charted in gm. per week). The food mixtures had the following composition.

	Rat 1684♀.		Rats 2961♂ and 2991♂.	
	Period I.	Period II.	Period I.	Period II.
	per cent	per cent	per cent	per cent
Casein	18	18	18.0	18.0
"Artificial protein-free milk" IV*.	29		29.5	29.5
Natural "protein-free milk"		28		
Starch	28	29	27.5	27.5
Butter fat	18	18	18.0	18.0
Lard	7	7	7.0	7.0
Yeast				0.1 gm. per day.

* The composition of "artificial protein-free milk" IV is given in *J. Biol. Chem.*, 1913, xv, 317.

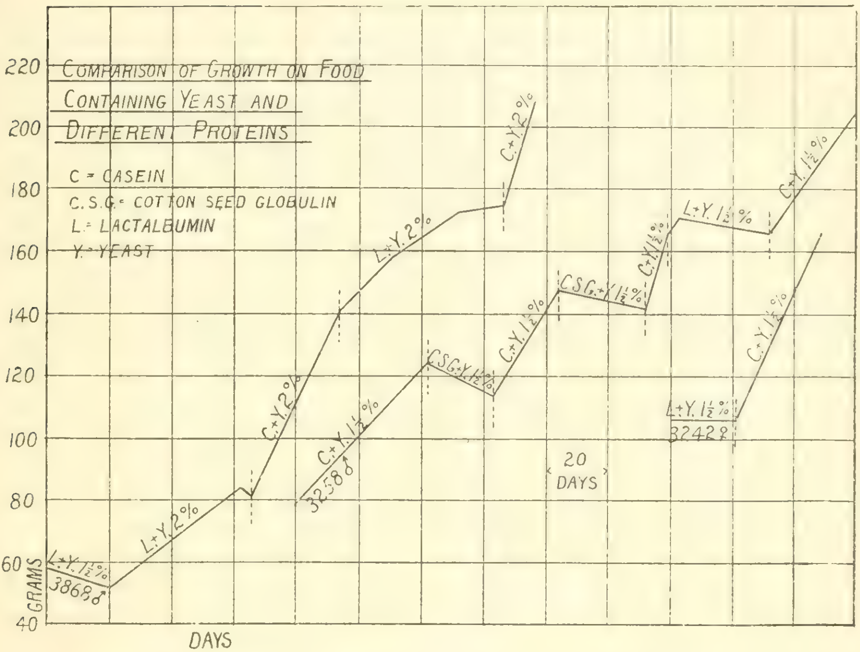

Chart II. Illustrative graphs showing the ready growth on casein food and the failure to grow well on lactalbumin food and cotton seed globulin food when yeast was used as a course of water-soluble vitamine along with "artificial protein-free milk." The food mixtures had the following composition.

	Casein food.	Cotton seed globulin food.	Lactalbumin food.
	per cent	*per cent*	*per cent*
Protein	18.0	18.0	18.0
"Artificial protein-free milk" IV	29.5	29.5	29.5
Starch	26.0–25.5	18.0	17.0–16.5
Butter fat	18.0	18.0	18.0
Lard	7.0	15.0	16.0
Yeast	1.5–2.0	1.5	1.5–2.0

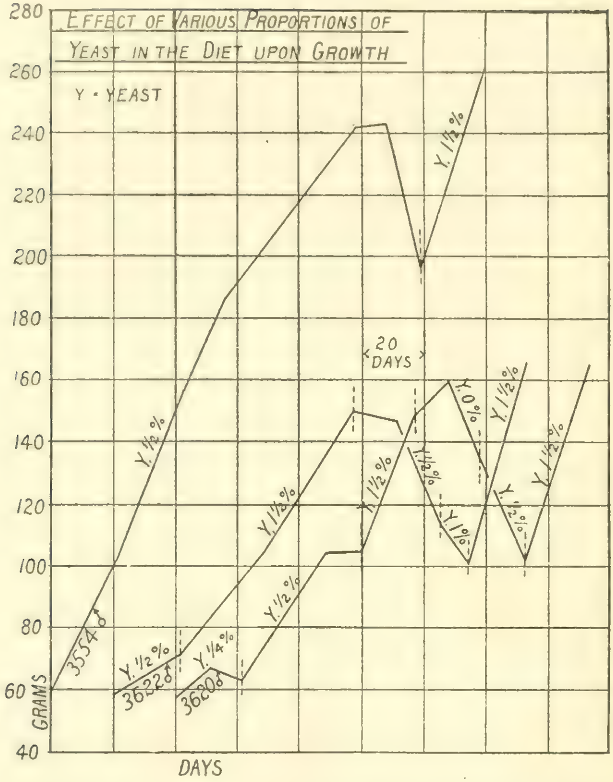

CHART III. Illustrative graphs showing comparative growth-promoting value of different proportions of yeast used as a source of water-soluble vitamine in the diet. The food mixtures had the following composition.

	per cent
Casein	18.0
"Artificial protein-free milk" IV	29.5
Starch	27.25–26.0
Butter fat	18.0
Lard	7.0
Yeast	0.25–1.5

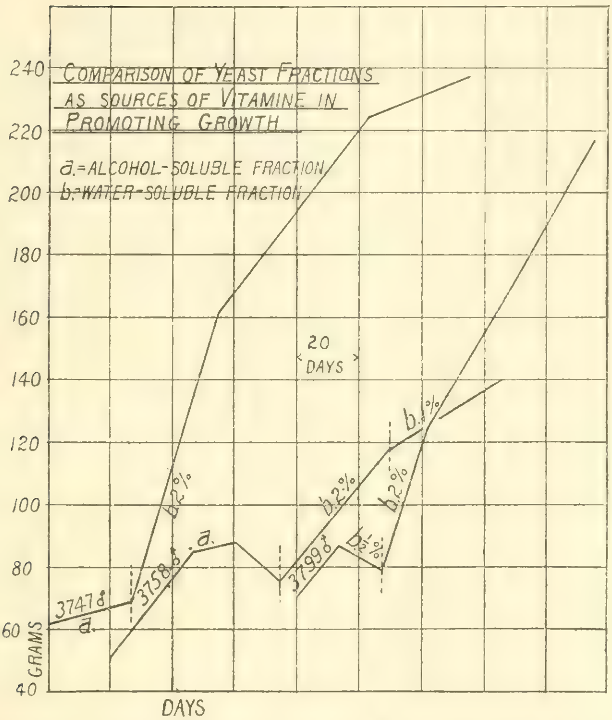

CHART IV. Showing a comparison of the efficiency of the alcohol-soluble fraction (*a*) and the water-soluble fraction (*b*) (described on p. 158) as a source of water-soluble yeast vitamine in promoting growth. The food mixtures had the following composition.

	per cent	per cent
Casein	18	18.0
"Artificial protein-free milk" IV	28	28.0
Starch	29	28.5–27.0
Butter fat	18	18.0
Lard	7	7.0
Alcohol-soluble fraction (*a*)	0.044	
Water-soluble fraction (*b*)		0.5–2.0

A NOTE ON MODIFICATIONS OF THE COLORIMETRIC DETERMINATION OF URIC ACID IN URINE AND IN BLOOD.

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(Received for publication, May 16, 1917.)

For the estimation of uric acid in urine, a rapid micro method such as the procedure suggested in the Benedict-Hitchcock modification[1] of the Folin-Macallum method has numerous advantages over the slower and more laborious Folin-Shaffer[2] method. A colorimetric method is especially useful where uric acid excretion is determined for short periods, involving the accurate determination of small quantities in order to place emphasis on slight variations in uric acid output. Loss by manipulation is reduced to a minimum. Previous attempts to use the Benedict method in these laboratories have, however, failed to yield accurate results, owing chiefly to rapid variations in the color of the unknown uric acid solutions. Benedict himself refers to this difficulty indirectly.

In speaking of the deeper color produced by a given quantity of uric acid when potassium cyanide has been added to the solution, he says: "This effect seems to be due chiefly to a marked diminution in the rate of fading of the color from solutions containing the cyanide." He adds: "The slower fading of the color under these conditions is a distinct advantage." Later he states that, due to the fact that reoxidation of the colored compound is accelerated by filtration, an unfiltered clear standard will read higher than the same solution after filtration. Myers and Fine[3] make note of the fact that the colors must be matched "*at once*" as they

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[1] Benedict, S. R., and Hitchcock, E. H., *J. Biol. Chem.*, 1915, xx, 619.

[2] Folin, O., and Shaffer, P. A., *Z. physiol. Chem.*, 1901, xxxii, 552.

[3] Myers, V. C., and Fine, M. S., The Chemical Composition of the Blood in Health and Disease, New York, 1915, p. 17.

"fade at a fairly rapid rate." Benedict directs the comparison of the unknown solution with a "simultaneously prepared" standard solution, assuming that the two solutions lose color at the same rate.

From our experience we have reached the conclusion that the rate of fading of the color is quite different in the unknown and standard solutions. In this case, any delay in reading, such as may be caused by the development of turbidity in either solution, will result in a considerable error.

We were led to a more detailed study of the rates of fading in the standard and unknown solutions by the following experiment.

A sample of urine was treated according to the procedure suggested in the Benedict uric acid method and the resulting unknown solution compared with a standard solution set at 20 mm. in the colorimeter. The unknown solution read at 17.5 mm. The solutions, when matched in color, were left in the colorimeter 20 minutes. The unknown was then noticed to be much lighter than the standard and was adjusted to match the standard at 19.9 mm. 10 minutes later the unknown was again found lighter than the standard and matched at 21 mm. This suggested that the standard solution was not fading at all or was fading at a much slower rate than the unknown solution. Both solutions were, therefore, left standing over night in closed flasks and were read against each other on the following day, 20 hours after they were made up. With the standard again set at 20 mm. in the colorimeter, the unknown matched at 44 mm. The old standard was then compared with a fresh standard containing the same amount of uric acid and set at 20 mm. The reading given by the 20 hour standard was 48 mm. The color of the unknown was too faint to compare in the colorimeter with the freshly prepared standard but it was calculated (20:44::48:X) that a depth of about 106 mm. would be necessary to match in color 20 mm. of the new standard.

Further experiments led to the belief that, although the rate of fading of the standard solutions varies slightly, under the conditions used by us, an allowance of 5 per cent loss of color in the standard per hour for the first 2 or 3 hours after maximal color has developed is probably justifiable. Thus, when the standard solution in the colorimeter is first set at 20 mm., it is moved to 20.5 mm. before reading a second unknown against it ½ hour later. By making this small correction, it is possible to read several unknown solutions against the same standard and, with attention to other factors discussed later in this paper, duplicate analyses have been obtained which checked very closely.

Benedict recommends a delay of $\frac{1}{2}$ minute after adding the reagents to the standard uric acid solution to allow maximum color development before diluting. We find that the development of color does not seem to be affected by dilution and that the maximum color is not attained for 15 minutes after the addition of the alkali to the standard uric acid solution. This is illustrated by the following experiments, in which different standards were read at intervals of 5 minutes against freshly prepared standards, diluted after standing $\frac{1}{2}$ minute, and *immediately* placed in the colorimeter at 20 mm. The readings were made as rapidly as possible, usually in less than 1 minute. The results are given below.

Standards.		
Fresh.	After	
mm.	*min.*	*mm.*
20	5	19.9
20	10	17.6
20	10	17.5
20	15	15.0
20	15	15.5
20	15	15.4
20	15	15.0

No further development of color occurred after 15 minutes. This led us to adopt the routine of allowing all standard solutions to stand 15 minutes before using.

The development and fading of color in the unknown solutions were next investigated and were found to vary greatly in different unknowns, even in duplicate analyses on the same urine. The data from such an experiment are given below. In each case the unknown solutions were compared with fresh standards, diluted to the mark after standing $\frac{1}{2}$ minute, and used *immediately*.

Standards.		Sample 1.	Sample 2.
mm.		*mm.*	*mm.*
20	Read at once.	9.5	9.0
20	After 5 min.	7.5	7.4
20	" 10 "	8.2	8.0
20	" 15 "	8.5	8.0
20	" 35 "	9.5	8.0

Since the lowest readings in duplicate analyses were found to check invariably within the limits of error in reading, we adopted the procedure of keeping each unknown solution in the colorimeter several minutes, making readings every 2 or 3 minutes, until the point of maximum color development had been reached. Although this point is reached in 5 minutes in each of the series of readings given above, samples have occasionally been found which required as long as 20 minutes for full development of the color.

Difficulty was sometimes caused by turbidity in the solutions. When the modified uric acid reagent devised by Benedict[4] was used, the solutions were seldom turbid. We found, however, that dilution with 20 to 30 cc. of water before adding the sodium carbonate solution was also of assistance in avoiding turbidity. In the very rare instances where the solutions did become turbid, a perfectly clear fluid was obtained by centrifuging for 1 or 2 minutes. This is more rapid than filtering as advised in the Benedict method and avoids the oxidation, with consequent loss of color, caused by filtration.

We have finally used the method as follows.

2 cc. of urine are measured into a centrifuge tube, diluted to about 5 cc. with water, stirred, and treated with twenty drops of ammoniacal silver magnesium solution.[5] The contents of the tube are well mixed with a stirring rod and the latter is washed down with water. The tube is then centrifuged 2 to 4 minutes. The supernatant liquid is poured off as completely as possible, the tube being inverted and the inside of the lip touched with a towel or piece of filter paper. Compressed air or suction is useful in removing the last traces of ammonia. The residue in the tube is then treated with two drops of 5 per cent potassium cyanide solution and the mixture thoroughly stirred with a slender stirring rod for half a minute. About 1 cc. of water is added and the solution is again stirred. 2 cc. of uric acid reagent are then added, the mixture is stirred, and washed into a 50 cc. flask with 20 to 30 cc. of water. After the addition of 10 cc. of 20 per cent sodium carbonate solution, the solution is diluted to the mark and compared in the colorimeter with a solution obtained by treating 5 cc. of standard uric acid solution (containing 1 mg. of uric acid) with two

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[4] Neuwirth, I., *J. Biol. Chem.*, 1917, xxix, 478, note 4.

[5] The composition of all solutions used may be found in the paper by Benedict and Hitchcock,[1] with the exception of the modified uric acid reagent described by Neuwirth.

drops of 5 per cent potassium cyanide solution, 2 cc. of uric acid reagent, 10 cc. of 20 per cent sodium carbonate solution, diluted to 50 cc., and allowed to stand 15 minutes before using. If any turbidity develops, it is removed by centrifuging. The unknown solution is placed in the colorimeter with as little delay as possible after it is made up and readings are taken every 2 or 3 minutes until maximum color development is reached, which usually occurs in 5 to 10 minutes. If the standard has stood more than 15 minutes, allowance is made for a loss of color of 5 per cent per hour. Standards are seldom used longer than 1 hour.

By this procedure uric acid added to urine has been determined quantitatively and duplicate analyses have been obtained which agree uniformly within per 2 cent, as shown by the following figures.

Volume of urine.	Uric acid.	Colorimeter readings.
cc.	*mg.*	*mm.*
1,080	346*	15.6
	348	15.5
1,005	283	17.7
	285	17.6
910	286	15.9
	286	15.9
1,350	307	22.0
	304	22.2

* These figures were all obtained from a subject who was on a purine-free diet.

In attempting to use the Benedict modification of the Folin-Denis method for determining uric acid in the blood,[6] even greater difficulty was experienced in obtaining consistent results. In addition to the errors caused by rapid changes of color due to development and fading of color in both the standard and unknown solutions, as discussed previously in this paper, the solutions were found to be tinged with yellow, instead of water clear, after adding colloidal iron and filtering. This yellow color could not be removed by adding salt solution or avoided by the addition of less iron. It apparently resulted in loss of the uric acid

[6] Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 629.

by oxidation, as very faintly colored or colorless solutions were usually obtained from these analyses on adding uric acid reagent and sodium carbonate solution. The loss of uric acid was roughly proportional to the amount of iron which had been added. On a few occasions only, in the course of many analyses, were results obtained in which there had probably been no loss of uric acid. Of known amounts of uric acid added to samples of blood, only 25 to 50 per cent were recovered.

The omission of the precipitation by colloidal iron was suggested by Dr. Louis Baumann,[7] who had experienced similar difficulties. By following this procedure, in which reliance is placed on the treatment with dilute acetic acid and heat for the removal of most of the protein, results have been obtained which agree within the limits of experimental error.

10 cc. of fresh oxalated blood are pipetted into a casserole containing 50 cc. of boiling 0.01 N acetic acid and heated to boiling until coagulation is complete. It is then filtered into a second casserole, washing the coagulum and the casserole in which coagulation took place with 200 cc. of boiling water. The filtrate, which should be almost if not entirely water clear, is evaporated to 50 cc. either on a water bath or over an asbestos mat with a small central hole for the free flame. This latter precaution is to avoid oxidation on the sides of the dish. When the volume of the solution is about 50 cc. it is washed quantitatively with boiling water into a 100 cc. casserole, after filtering if there is any precipitate. This solution is then concentrated to about 10 cc., washed with hot water into a centrifuge tube, 2 cc. of silver magnesium mixture are added, and the whole is well stirred with a slender glass rod. It is advisable at this point to allow the mixture to stand at least 1 hour as the uric acid precipitate often forms quite slowly. After centrifuging, the supernatant liquid is carefully removed and the same procedure outlined under the determinations on urine is followed for preparing and reading the colored solution. A standard containing 0.5 mg. of uric acid, diluted to 50 cc. and set at 20 mm. in the colorimeter, gives about the right depth of color for matching against a normal blood, if a 10 cc. sample has been taken and the resulting colored solution made up to 25 cc. In the case of bloods containing larger amounts of uric acid, the unknown may be diluted to 50 cc. or a standard containing 1 mg. of uric acid in 50 cc. may be used.

The results of a few analyses are given below to show that,

[7] The writer desires to express gratitude to Dr. Louis Baumann, Iowa State University, for a number of helpful suggestions based on work done by Mr. Thorsten Ingvaldsen and received by private communication.

after endeavoring to control all the variable factors outlined above, accurate and consistent results have been obtained. Some of the samples were from pathological cases.

10 cc. of blood.	0.93 mg. of uric acid.
10 " " " + 1 mg. of uric acid.	1.94 " " " "

Volume of blood sample.	Uric acid per 100 cc.
cc.	*mg.*
10	2.4
10	2.5
10	3.0
10	2.8
10	4.2
10	4.2
10	7.4
5	7.5
5	3.1
10	3.0

THE NUTRITIVE VALUE OF THE DIAMINO-ACIDS OCCURRING IN PROTEINS FOR THE MAINTENANCE OF ADULT MICE.*

By E. M. K. GEILING.

(*From the Department of Animal Husbandry, University of Illinois, Urbana.*)

(Received for publication, May 25, 1917.)

Review of the Previous Work on the Nutritive Value of the Diamino-Acids Occurring in Proteins.

The functions of the different amino-acids in the animal body have been studied mainly by means of three general methods. The first consists of feeding completely hydrolyzed proteins—hydrolysis may be effected by either acids or enzymes—from which the amino-acids whose functions are to be ascertained have been removed. This method has been developed and extensively used by Abderhalden and associates who have obtained some very valuable results. More recently Hopkins and coworkers have employed this method with considerable success.

According to the second method rations made of pure proteins lacking or deficient in one or more amino-acids are fed to animals. The behavior of the experimental animals on such a ration will serve as a good index of the function of the missing or deficient amino-acids. Osborne and Mendel and associates (among others) have pursued this line of investigation and have secured results of great significance and value to the science of nutrition.

The third method of attacking this problem has been used by Abderhalden and more recently by Mitchell;[1] it consists in supply-

* The results presented in this paper formed part of a thesis submitted to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Animal Husbandry.

ing the nitrogenous portion of the ration with pure amino-acids. This method promises to be very successful, if the technique is well developed. The main difficulty seems to be that of securing a sufficient consumption of the food for considerable periods of time. At present, however, its application must be somewhat limited on account of the high cost of the amino-acids.

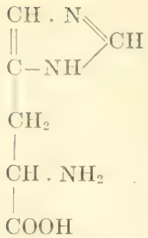
Much of the earlier work on the indispensability of certain of the amino-acids to the animal organism is of little value, because the rations used were inadequate in the nutrients other than protein. For example, in the literature one finds rations used composed only of hydrolyzed protein and cane sugar. Obviously, the poor results obtained with such a food mixture may be due just as much to the absence of the accessory substances, mineral salts, or fat as to the deficiency in the protein make-up.

Henriques and Hansen,[2] using the first named method, removed the diamino-acids from an enzyme digest of a protein by precipitation with phosphotungstic acid; after removal of the excess of the reagent from the filtrate, the product was evaporated to dryness *in vacuo*. The resultant mixture contained presumably only monoamino-acids and formed the sole source of nitrogenous material for their feeding experiment. Only one rat was used in their experiment. The authors concluded from this single trial that the diamino-acids could be entirely dispensed with by the animal organism. Mitchell[1] aptly criticizes this work in the following words: "Unfortunately the particular protein used was not mentioned, and the lack of all details of the phosphotungstic acid precipitation precludes any attempt to judge of the completeness of separation of monoamino- from diamino-acids. The experiment lasted only 26 days. During the last 17 days only were positive nitrogen balances obtained and they were such that they could hardly be said to constitute convincing evidence of the nutritive adequacy of the ration fed, especially in view of the fact[3] that the determination of the total urinary nitrogen per day of rats, with the exercise of the utmost care as to collection and preservation, is subject to errors of 10 per cent or more, due to incomplete collection. The body weight increased slightly during the last half of the experiment, but during the last 3 days it declined slightly but consistently. It is unfortunate that the experiment was not continued further to determine whether this final decrease was significant or not. This criticism is especially justified by the many experiments that may be quoted on the feeding of synthetic rations, in which entirely erroneous conclusions may be drawn if attention is confined to the first 20 or 30 days of observation."

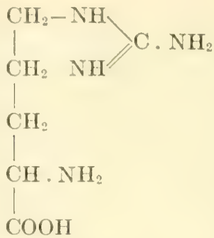
Abderhalden[4] in a recent extensive communication discusses the bio-

logical value of the amino-acids and presents some very interesting material. He has succeeded in obtaining a completely hydrolyzed mixture of amino-acids from meat by digestion first with enzymes and finally with 10 per cent sulfuric acid. No trace of any intact polypeptide compound could be discovered. On this mixture as the sole source of nitrogen a dog lived in good health and increased about 10 kilos in weight for 100 days. Lysine was removed from the hydrolyzed product and resulted in a negative nitrogen balance. When hydrolyzed gliadin formed the sole source of nitrogen, a negative balance was also obtained. Even with the addition of lysine to the hydrolyzed gliadin, a positive balance could not be secured, although an improvement was observed. The test periods lasted only 7 days. The results obtained with arginine were inconclusive, as the experiments were discontinued before an after-period with arginine was completed. It is suggested that ornithine can replace arginine, but the evidence for this is not very good, as Abderhalden admits. From the experiments with histidine and cystine no definite conclusions were drawn. Histidine was removed with $HgCl_2$ and the resultant amino-acid mixture gave a negative nitrogen balance. Addition of histidine had not the effect of restoring a positive balance. From this Abderhalden concludes that the removal of histidine must have brought about a change in the amino-acid mixture, probably destroying or rendering inactive some of the essential amino-acids. Cystine is regarded as probably being an essential amino-acid. Its removal was effected with acetic acid, but was not quantitative. The excess acetic acid could not be evaporated without destroying some of the amino-acids. Here again the amino-acid mixture, when supplemented with cystine, was inadequate. Considerable difficulty was experienced in some cases in getting the animals to eat the food. Other animals again vomited their food or contracted diarrhea. Probably the chief criticism against this work is that the experimental periods were of too short duration, usually only 7 days with any one ration.

Ackroyd and Hopkins[5, 6] recently have made some interesting contributions to our knowledge concerning the function of some of the amino-acids in the animal body. Rats were used as experimental subjects. The nitrogen requirements were supplied by casein, completely hydrolyzed with 25 per cent sulfuric acid. The amino-acids to be studied were removed from this hydrolyzed product and the resultant mixture, supplemented with cystine and tryptophane, formed the sole source of nitrogen in the rations used. Rats fed a ration from which the arginine and the histidine had been removed by the Kossel and Kutscher method ceased to grow and lost weight. When these amino-acids were replaced, growth was resumed at a normal rate. Further experiments showed that the animals grew when either arginine or histidine was present, thus indicating that these two amino-acids are interchangeable in nutrition. The close similarity between these two amino-acids is shown by writing their structure in the following manner:



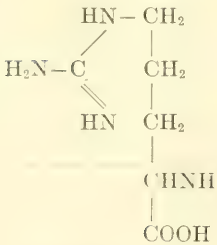
Histidine.
(Iminazole-amino propionic acid.)



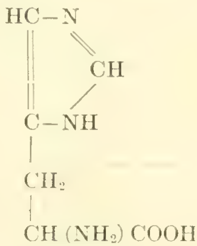
Arginine.
(Guanidine-amino propionic acid.)

"The essential molecular changes involved in passing from one form to the other—the opening or closing of a ring and the addition or removal of an amino group—may be inferred from our general knowledge of the chemical powers of the body, and may be regarded as essentially physiological processes."

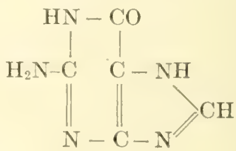
In another series of experiments these investigators have shown the special part played by arginine and histidine in purine metabolism. A comparison of the structural formula of these two amino-acids with that of guanine, a typical purine base, will show their close relationship.



Arginine.



Histidine.



Guanine.

Allantoin is the main end-product of purine metabolism in rats. Quantitative determinations of allantoin in the urine of rats will, therefore, reveal the operation of any factors which have influenced purine metabolism. In this connection the following experiments were conducted: Rats were first fed a complete amino-acid mixture to determine the normal allantoin excretion; then arginine and histidine were withdrawn in a second period, and were again replaced in a third period. A marked decline in the allantoin excretion was noted during the period when arginine and histidine were withdrawn from the ration. To obviate a criticism that the falling off in allantoin is due to a general lowering of metabolism, owing to the removal of a factor essential to nutrition, two further experiments were performed to settle this point in the following manner: Rats were fed a ration from which the tryptophane, which is essential for maintenance, had

been removed, and the allantoin was determined in the urine. The animals declined in weight, but the allantoin excretion remained unchanged, thus indicating that arginine and histidine function in a special way in purine metabolism, and that the decline in allantoin during the second period was due to a removal of these amino-acids, and not to a lowering of the general metabolism. A similar decline in weight with unchanged allantoin excretion was noted, when the vitamine supply of the ration was withdrawn.

Previous to this work Abderhalden and Einbeck[7] studied the relationship between histidine and purine formation in the animal organism. They supplemented a complete ration with histidine and obtained no increase in uric acid or allantoin excretion. Later these experiments were repeated in conjunction with Schmid,[8] but again negative results were obtained. In these experiments only the total nitrogen and allantoin were determined in the urine. Kowalevsky[9] also studied the rôle of histidine in the metabolism of a dog. A dog was fed with milk, bread, and cane sugar, and later the ration was supplemented with histidine. No increase in uric acid was noted in this latter period. There was an increase in urea and ammonia, and the urine was acid. The disagreement of this earlier work with that of Ackroyd and Hopkins can probably be accounted for by a difference in experimental procedure. The former workers fed histidine in addition to a complete ration, whereas the latter authors removed histidine completely from the diet and then noted the effect. This method is certainly the better one, and results secured by employing it are more significant than those obtained when the amino-acid was practically fed in excess.

Osborne and Mendel,[10] using the second general method of experimentation, namely, feeding purified incomplete proteins and then supplementing them with the amino-acids deficient or lacking in the ration, have made some important contributions to our knowledge of the physiological action of certain amino-acids. Lysine was shown to be essential for growth in rats. This was done by feeding gliadin, the alcohol-soluble protein of the wheat kernel, as the sole protein of a ration. The animals maintained their body weight for long periods of time, but failed to grow normally. On the addition of lysine to the ration normal growth was resumed. Similar results were obtained when other proteins low or lacking in lysine, like zein of maize, were used. Subsequently [11] these investigators have shown that for the normal growth of rats the rations used by them must contain somewhat over 2 per cent of the protein as lysine.

Buckner, Nollau, and Kastle,[12] using chickens as experimental subjects, fed them rations presumably high or low in lysine. The chicks were fed complex mixtures, such as wheat, wheat bran, sunflower seed, hemp seed, and skim milk, a ration taken as high in lysine, and a mixture of barley, rice, hominy, oats, and gluten flour, a ration taken as low in lysine. The animals receiving the first named ration grew normally, while those fed the latter remained stunted. From these results the authors conclude that the marked differences shown by these two lots of chicks in the rate of growth and development are due to "differences in the amino-acid content

of the two rations and in all probability to differences in the lysine content." This work is, however, open to serious criticism. It may be shown that their method for determining lysine in feeds is thoroughly unreliable. Furthermore, the grain mixtures fed the two lots of chickens differed so radically that the interpretation of their results is difficult.

Osborne and Mendel[13] have lately obtained results similar to those of Buckner, Nollau, and Kastle with chicks, but instead of using mixtures of grains, corn gluten, containing about 1 per cent of lysine, and a mixture of equal parts of corn gluten and lactalbumin, yielding about 10 per cent lysine, were used to supply the protein requirements. The balance of the rations was made up with protein-free milk, starch, butter fat, and lard. The data show in a striking way the difference in the efficiency of the two rations used. After 55 days the chick receiving the corn gluten ration gained only 52 gm., while the other chick receiving the corn gluten and lactalbumin ration gained 283 gm. The stunted chick exhibited no signs of malnutrition other than failure to grow.

However, McCollum, Simmonds, and Pitz,[14] in experiments on rats designed to ascertain the supplementary relationships among the naturally occurring foodstuffs, are forced to the conclusion that "in the protein mixture of the maize kernel and the oat kernel, lysine certainly is not the essential protein cleavage product which is present in amount so small that it is the limiting factor which determines the biological value of the proteins of these seeds." Thus, zein, though lacking in tryptophane and lysine, supplements the proteins of the oat kernel in a surprisingly efficient manner. Also, gelatin with its high lysine content does not improve the proteins of the maize kernel.

Rats fed a ration in which zein, supplemented with lysine and tryptophane, was the sole source of protein, did not always grow as rapidly, nor was growth as prolonged as was expected by Osborne and Mendel. Hence, in a subsequent experiment arginine was also added to the same ration (zein being very low in arginine) and resulted in improved growth. Histidine was added in one case, in addition to the arginine, and a slight increase in the growth was noted, but Osborne and Mendel[15] do not regard this as significant, inasmuch as another rat grew quite as rapidly in the absence of this amino-acid. This conclusion appears justified by the work of Ackroyd and Hopkins quoted above, in which it was shown that arginine and histidine seem to be interchangeable.

Myers and Fine[16] studied the effect of feeding rations low and high in arginine on the creatine content of rat muscle. The animals fed the "Osborne and Mendel" ration, low in arginine, contained about 2.5 per cent less creatine than did those fed on a diet rich in arginine. The fact that both arginine and creatine contain the guanidine ring makes it probable that the latter may be derived from the former in the body. When these data are submitted to a statistical examination, the differences become much more striking, and hence more significance may be attached to the conclusions than is done by the authors themselves.

Concerning the nutritive value of cystine, Osborne and Mendel[17] re-

port results showing that when proteins low in cystine, such as casein, are supplemented with this amino-acid, a much smaller amount of the protein is required in the ration to produce normal growth. For example, rats grew normally on a ration containing 15 per cent of casein, but when the protein was reduced to 9 per cent, growth was promptly limited. The addition of isolated cystine to the ration with 9 per cent casein at once rendered the ration decidedly more adequate for growth.

"Growth can be facilitated or repressed at will by the addition or withdrawal of the extra cystine from the diet containing 9 per cent of casein." These results at least suggest strongly that cystine too may be made a limiting factor in growth.

From the works just reviewed it will be seen that, with the exception of the work of Henriques and Hansen, all the data secured on the nutritive value of the diamino-acids were obtained with growing animals. It appears that lysine and cystine may be considered as essential for the growth of animals and that either arginine or histidine must be present to render the ration adequate for maintenance.

Preparation of the Food Materials.

In synthetic feeding experiments it is essential that the food materials used should be as pure as possible, for sometimes impurities present in even small amounts may have a very decided influence. Below are given, in brief, the methods used in the preparation of the different nutrients.

Casein.—Prepared from a solution of skim milk powder in twelve parts of water by repeated precipitation with dilute hydrochloric acid and solution in dilute sodium hydroxide. The product was finally extracted with alcohol and ether, dried, pulverized, and put through a 40 mesh sieve.

Dextrin.—According to the method of McCollum and Davis,[18] high grade corn starch was moistened with 0.5 per cent citric acid solution and made into a stiff paste which was heated in an autoclave at 15 pounds' pressure for 3 hours. The resultant gelatinous mass was cut into slices, washed with alcohol, dried, ground in a mill, and passed through a 40 mesh sieve.

Butter Fat.—Best quality creamery butter was melted at a low temperature (40°C.) and then centrifuged for 1 hour. The clear liquid was syphoned off and allowed to harden.

Protein-Free Milk.—In most of the experiments reported in this paper, the protein-free milk was prepared according to the method of Osborne and Mendel.[19] In some of the later experiments, a slight modification of this method was used in that a second filtration was introduced after neutralization of the filtrate from the lactalbumin. While this filtration removes some calcium phosphate, it also reduces the nitrogen content of the final product by about 0.1 per cent. In previous work done in this labora-

tory[20] it was shown that this modified preparation is adequate to cover the mineral requirements of adult mice.

Hydrolyzed Casein.—Two preparations of this product were used—one digested with enzyme for 2 months and the other for $3\frac{1}{2}$ months. 200 gm. of casein (either purified or commercial) and 20 gm. of pancreatin were mixed with 2 liters of water made slightly alkaline with ammonia, and kept in an oven at 40°C. for the periods mentioned above. The mixture was stirred every day. Toluene was added to prevent bacterial growth. At the end of each month 10 gm. of pancreatin were added. At the conclusion of the digestion period, the mixture was boiled and filtered. The precipitate was washed with boiling water, and the volume of the filtrate reduced to about 2 liters by evaporating on a water bath at a low temperature. Nine and a half volumes of redistilled 95 per cent alcohol were added to precipitate all the peptones, proteoses, and complex peptides. How complete this precipitation with alcohol is, is not known. The solution was filtered and the alcohol distilled off at a low temperature. The precipitate was washed with boiling water to dissolve out the tyrosine. The washings thus obtained were allowed to cool, and the tyrosine crystallized out. The tyrosine was washed with ice water, dried, and added to the material obtained from the alcoholic filtrate. The resultant product, when dried, was a brown powder, with a sharp taste, and did not give the biuret test (showing that only the very simplest peptides could be present). The amino nitrogen before and after hydrolysis of this product was determined according to the method of Van Slyke[21] in order to arrive at an estimate of the amount of peptide nitrogen present. The product hydrolyzed for 2 months contained 36.2 per cent peptide nitrogen, and the one hydrolyzed for $3\frac{1}{2}$ months, 24.25 per cent.

Monoamino-Acid Mixture.—About 50 gm. of hydrolyzed casein, prepared as outlined above and containing 24.25 per cent peptide nitrogen, were dissolved in 5 liters of 5 per cent sulfuric acid. Phosphotungstic acid, dissolved in 5 per cent sulfuric acid, was added in excess to precipitate the diamino-acids, and any peptides which may have a diamino-acid component.[22] The mixture was allowed to stand at room temperature for 48 hours, after which it was filtered. The excess phosphotungstic acid and sulfuric acid were removed from the filtrate with barium hydroxide. The solution was filtered and evaporated to dryness at a low temperature. The product thus obtained was of a light yellow color and had a fairly sharp taste. The peptide nitrogen in this preparation was 23.54 per cent.

To test whether this monoamino-acid mixture contained any diamino-acids in peptide form, the following experiment was carried out. A sample of the mixture was completely hydrolyzed with 20 per cent hydrochloric acid, the acid removed by evaporation on the water bath at a low temperature, and the residue taken up with water. The solution was filtered to remove the melanin, of which there was very little, made up to volume, acidified with hydrochloric acid, and phosphotungstic acid was added, according to the directions as outlined by Van Slyke.[21] No precipitate was formed; only a slight scum floated on the surface, which was not a

phosphotungstate of the diamino-acids, since it was insoluble in boiling water. This shows conclusively that the diamino-acids were all removed from the monoamino-acid mixture in the first precipitation with phosphotungstic acid, even those that may have been present in peptide form.

Diamino-Acids.—The diamino-acids used were obtained from gelatin by hydrolyzing with 25 per cent sulfuric acid for 40 hours on an electric sand bath, then diluting the mixture with water to give a 5 per cent sulfuric acid solution. Phosphotungstic acid dissolved in 5 per cent sulfuric acid was added in excess, and the mixture was allowed to stand at room temperature for 48 hours, and filtered. The precipitate was washed with a 2 per cent sulfuric acid solution of phosphotungstic acid, decomposed with barium hydroxide, and the mixture was filtered. The excess alkali was removed with sulfuric acid, and the light colored solution was again filtered and evaporated to dryness at a low temperature.

Other Amino-Acids.—Cystine was prepared from wool according to Denis'[23] modification of Folin's method.[24] The Hopkins-Cole method was used in the preparation of tryptophane from casein. Arginine, histidine, and lysine were obtained from gelatin, blood meal, or casein according to the method of Kossel and Kutscher.

EXPERIMENTAL.

The experiments about to be reported were all conducted with adult mice for the purpose of ascertaining whether or not the diamino-acids, arginine, histidine, and lysine, which are precipitated with phosphotungstic acid in acid solution, are necessary for the maintenance of adult mice. In all the experiments with hydrolyzed protein products it was assumed that cystine is an indispensable amino-acid for the maintenance of mice. Therefore such preparations, whether complete or containing only monoamino-acids, were supplemented with cystine in the large majority of cases. Some evidence will also be presented confirming the correctness of this assumption.

The method of attacking this problem was to feed hydrolyzed casein, prepared as described above minus the diamino-acids precipitable by phosphotungstic acid in acid solution. This preparation—"monoamino-acid mixture"—generally supplemented with tryptophane and cystine, was fed in an otherwise adequate non-nitrogenous ration, except for the small amount of nitrogen occurring in protein-free milk. In other words, the diamino-acids were made the limiting factor in an otherwise complete ration. If such a food mixture is sufficient to cover the maintenance requirements, the conclusion of Henriques and

Hanson is justified. The body then is able to synthesize the diamino-acids required. On the other hand, if this ration proves inadequate, it would tend to show that the material precipitated by the phosphotungstic acid in acid solution is necessary for the maintenance of adult mice.

In this investigation the problem was somewhat complicated by the fact that the casein was not completely hydrolyzed, from which it may be argued that some of the essential monoamino-acids may have been completely precipitated in peptide linkage by the phosphotungstic acid. Consequently the failure of maintenance may be due just as much to the absence of these essential monoamino-acids, as to the lack of the diamino-acids. To ensure the presence of sufficient tryptophane, it was always added to the rations, except in one case. Should it, however, be shown that this "monoamino-acid mixture," supplemented only with tryptophane and the diamino-acids, is adequate for maintenance, then the above objection becomes void. The next step was to ascertain whether all or only some of the diamino-acids are necessary for maintenance of adult mice.

Before the main problem of the investigation could be taken up, several preliminary points had to be settled. These will be discussed in their logical order. The details of the experimental procedure were similar to those followed by Mitchell,[1] except that some of the rations used were made into a biscuit form.

The biscuit was prepared by thoroughly mixing the dry ingredients and adding enough warm water to form a stiff mush. This mush was dried in a thin layer on glass plates at a low temperature and subsequently the brittle mass was broken in pieces of convenient size. A small tin plate, about 3 inches in diameter, was placed below the porcelain crucible containing the food for the purpose of catching any of the ration which might be thrown out of the crucible by the mice. Considerable difficulty was experienced in getting some of the experimental animals to consume a sufficient amount of a few of the rations fed. In these cases the mice scattered the food but most of it fell on the plate below, and could be recovered and fed again, if it was not soiled. In such instances the food intake measurement must be regarded as an approximation only.

Experiment 1.—The first step was to compound a ration which would serve as an intermediate step between the grain and the experimental rations—a preliminary ration—and also ascertain the percentage of casein necessary for maintenance. For this purpose the following ration was made into a biscuit form.

Ration 1.

	per cent
Skim milk powder (6.01 per cent nitrogen, or $6.01 \times 6.33 = 38.043$ per cent protein) yielding 10.65 per cent protein in the ration.	28
Butter fat	5
Lactose	10
Dextrin	57

All the ingredients were passed through a 40 mesh sieve before being mixed.

The details concerning the weight of animals and duration of the experiment are appended:

Days.	No. 71a ♀.	No. 72b ♀.	No. 71b ♀.	No. 72 ♀.	No. 62 ♂.	No. 62a ♂.
	gm.	gm.	gm.	gm.	gm.	gm.
1	26.4*	18.7	26.7	25.2	21.2	24.4
7	19.2	17.5	23.5	23.7	19.5	23.9
14	17.5	15.7	19.4	22.6	21.0	26.5
28	15.8	15.7	19.8	22.0	19.5	26.0
35	16.8	15.5	22.6	22.8	19.7	26.3
42	17.5	14.8	22.7	23.5	20.0	26.2
49	17.6	15.5	22.1	23.4	20.0	26.8
56	17.1	16.2	21.8	23.7	19.5	28.5
63	17.2	16.3	22.1	23.4	19.0	28.5
70	17.6	16.6	22.4	23.1	18.2	28.5
77	17.7	17.5	24.0	23.9	20.7	28.3
84	16.8	17.2	23.5	22.5	20.2	26.7
91	17.3	17.5		24.0**	21.2**	27.2**
98	19.0	18.7				
105	18.8	19.0				
112	18.0	19.2				
119	19.7	18.7				
126	18.2	18.5				

* Gave birth to young but ate them. **Weight on 88th day.

From these figures it will be seen that Mice 71a ♀ and 72b ♀ maintained themselves satisfactorily for 126 days. The other four mice showed satisfactory maintenance at a higher weight for 84 to 88 days. In every case there was a drop in weight during the first 7 days. Such a drop in weight was observed in practically every experiment in this investigation following a change in ration. The food intake was quite satisfactory. Each

animal was fed about 2 gm. of ration per day. All the animals used in the present investigation were placed on this ration for several weeks before being used for an experiment. In later work this preliminary ration was somewhat altered, more butter fat and lard being added.

Experiment 2.—It was thought advisable to test whether adult mice could be maintained on a lower protein diet. For this purpose the following ration was made up:

Ration 2.

	per cent
Dextrin	55
Lactose	15
Sucrose	5
Butter fat	5
Skim milk powder—equivalent to 7.6 per cent protein in the ration	20

Four mice were fed this ration and took the food satisfactorily.

Days.	No. 74 ♂.	No. 74a ♂.	No. 75 ♀.	No. 75a ♀.
	gm.	*gm.*	*gm.*	*gm.*
1	21.8	24.9	29.7	17.2
7	17.9	20.0	26.2*	19.2
14	16.2	18.2	20.2	19.7
21	15.0	17.2	20.8	20.7
28	15.2	17.5	20.7	18.5
35	15.3	18.7	20.6	19.3
42	15.2	17.5	18.0	18.1

* Gave birth to young but ate them.

Since maintenance of weight on this ration did not appear to be as satisfactory as on Ration 1, it was decided not to reduce the protein of any of the rations below 10 per cent. These results are in agreement with those obtained by Wheeler[25] who found that growing mice require a higher per cent of protein than rats, which are able to maintain themselves on a very low per cent of protein.

Experiment 3.—A further test was made to ascertain whether a ration composed of 10 per cent purified casein was adequate for the maintenance of full grown mice. The ration used was made up as follows:

Ration 3.

	per cent
Purified casein	10
Butter fat	5
Protein-free milk	28
Dextrin	57

Four adult female mice were placed on this ration, with results as shown below. Before being placed on this ration, the mice were fed Ration 1 for 19 days.

Days.	No. 30 ♀.	No. 30a ♀.	No. 35 ♀.	No. 35a ♀.
	gm.	*gm.*	*gm.*	*gm.*
1	24.2	25.2	25.0	20.5
7	24.4	24.3	23.7	20.0
14	24.3	24.8	24.8	22.6
21	24.2	25.2	24.8	22.2
28	22.2	24.2	24.0	21.0
35	24.7	25.7	25.3	22.4
42	23.5	25.9	25.6	21.7**
49	28.0	28.0	25.2	21.8
51	23.5	27.0*	26.5*	21.5

* Eaten by a cat.

** Affected with lice; isolated and treated with carbolated vaseline.

From these data it is obvious that Ration 3 satisfies the purpose for which it was compounded, at least for periods of 51 days. The casein of this ration was replaced by hydrolyzed casein in Experiment 6.

Experiment 4.—In this investigation casein and its derivatives had to be subjected to heat; hence it was considered advisable to know the effect of heating on the nutritive value of these compounds.

For this purpose purified casein was suspended in nitrogen-free water and boiled in a casserole on an open flame for 2 hours. Thereafter the water was evaporated on a water bath at a low temperature. Finally the

"boiled" casein was dried in an electric oven at 40°C. Ration 4 was made up as follows:

Ration 4.

	per cent
"Boiled" casein	10
Protein-free milk	28
Butter fat	5
Dextrin	57

The ingredients were made up into a biscuit form, as described above. Four mice were fed this ration after having been on Ration 1 for 26 days.

Days.	No. 60 ♀.	No. 60a ♀.	No. 53 ♀.	No. 53a ♀.
	gm.	*gm.*	*gm.*	*gm.*
1	18.0	18.0	21.0	18.0
7	17.5	18.0	20.3	19.7
14	18.4	18.2	17.7	20.0
21	22.0	20.6	19.0	21.3
28	21.9	20.3	19.9	21.3
35	20.5	19.0	21.4	19.2
42	23.2	20.7	20.0	20.0
49	23.2	21.8	21.8	22.2

These results indicate that casein suspended in water for 2 hours at 100°C. does not lose its nutritive value.

Experiment 5.—This experiment was planned to determine the effect of heating moist casein in the autoclave for 1 hour at 15 pounds' pressure. McCollum and Davis[26] recently reported that the nutritive value of casein is affected by heating it in this way. Their experimental animals were young growing rats.

Ration 5 was made into a biscuit form out of the following ingredients:

Ration 5.

	per cent
Casein heated in an autoclave at 15 pounds' pressure for 1 hour	10
Protein-free milk	28
Butter fat	5
Dextrin	57

The four mice used in Experiment 4, together with four additional ones, were placed on this diet with the following results:

Days	No. 60 ♀.	No. 60a ♀.	No. 53 ♀.	No. 53a ♀.	No. 81 ♀.	No. 81a ♀.	No. 83 ♀.	No. 83a ♀.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	23.0	21.8	21.8	22.2	25.0	23.5	24.5	19.5
7	21.5	21.8	20.7	23.2	23.5	22.5	24.3	17.5
14	22.5	21.8	20.6	22.7	24.2	23.6	23.5	16.5
21					23.5	22.4	22.7	
28	22.2	21.0	20.9	21.1	21.0	21.5	21.5	19.3
35	21.8	21.3	20.7	21.8	17.0	19.3	21.0	19.4
42	21.2	22.7	18.5	21.7	15.6*	18.4*	19.0**	17.5**
49	21.3	22.3	19.0	21.5	14.6	19.2	22.0	16.3
56	19.5	21.5	19.5	20.5	Died.	19.3	22.7	15.0
63	19.0	21.7	19.2	21.0	52nd	19.0	23.3	15.0
70	19.6	20.2	19.1†	21.0†	day.	18.0	22.0	15.5
77	19.7	20.6	21.5	22.8		17.6	20.0	15.0
84	16.8	19.4	21.5	24.0		12.2‡	18.0	12.2
91	Died	16.3	21.7	24.2		Died	16.0	10.7
98	92nd	Died				84th	18.0	10.1
	day.	91st				day.	Died	Died
		day.					101st	98th
							day.	day.

* Added cystine and 10 per cent lard.

** Changed to unboiled casein and 10 per cent lard.

† Butter fat containing casein added.

‡ Weight on the 79th day.

After Nos. 53 ♀ and 53a ♀ had been on the autoclaved casein ration for 70 days, they were fed a ration made of butter fat containing some untreated casein. The impurity of this butter fat was not discovered until the 21st day of feeding. The animals immediately gained in weight, thus showing the beneficial effect of the addition of only a small amount of unheated casein. With these subjects this ration was discontinued on the 91st day of observation. The remaining six animals gradually lost in weight and in each case ultimately died, thereby verifying the conclusion of McCollum and Davis that heating casein in an autoclave for 1 hour at 15 pounds' pressure destroys its nutritive value. The decline in weight, however, was not as rapid as in the case of the growing rats in their experiments. All the experimental mice were fed 2.5 gm. a day and took the food well, except in the declining stages, when the food was much scattered.

The addition of cystine to the ration of Mouse 81 ♀ on the

42nd day of observation exerted no beneficial effect, the mouse declining in weight steadily and dying on the 52nd day. A simultaneous addition of cystine to the ration of Mouse 81a ♀ was accompanied by a slight increase in weight, and maintenance of weight for 3 weeks, although ultimately this animal died also. With Mouse 83 ♀ a change to a ration containing unheated casein on the 42nd day caused a rise in weight for 3 weeks, but for reasons unknown a subsequent decline and death on the 101st day ensued. A similar change in the ration of Mouse 83a ♀ did not materially check the decline of this animal, which died on the 98th day of the test. The cause of the nutritive failure in these latter two cases may have been due to the unusually warm weather prevalent at that time.

Experiment 6.—The mice from Experiment 3 were placed on a ration in which the purified casein was replaced by a hydrolyzed casein preparation digested for 2 months with pancreatin, and further treated as described above. The product contained 36.2 per cent peptide nitrogen, but did not give the biuret test, showing that the peptides were of the very simplest kind. The ration fed was made into a biscuit form and contained the following materials:

Ration 6.

	per cent
Hydrolyzed casein (2 months)	10
Sucrose	5
Butter fat	5
Protein-free milk	28
Dextrin	52

The sucrose was added partly to overcome the sharp taste of the hydrolyzed casein. Later the sucrose was increased to 10 per cent. It is to be noted that the hydrolyzed casein in this experiment was not supplemented with tryptophane. The results obtained are given in the following table.

A decline in weight will be noted in all cases, indicating that the hydrolyzed casein lacked or was deficient in some essential constituent. This substance was either destroyed or removed from the digested casein, either in the undigested portion or in the precipitate from the nine and a half volumes of alcohol. Another possibility is that it was not present in sufficient quantity relative to the actual food intake to satisfy the animals' requirements. This latter statement appears to be the most probable one, for some mice did maintain themselves for a while, notably

Days.	No. 30 ♀.	No. 71b ♀.	No. 72 ♀.	No. 72b ♀.	No. 71a ♀.	No. 77 ♀.	No. 35a ♀.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	23.5	23.9	23.6	18.5	18.2	23.8	21.5
7	22.0	23.3	21.2	17.5	17.0	21.4	18.5
14	25.7	23.5	22.0	17.0*	17.3*	20.8§	18.4
21	24.5	22.0	20.5	16.0	15.0	20.0	18.0
28	24.0	21.3	22.0	15.2§	14.6§	19.5***	18.2
35	23.3	21.7	23.1*	14.8***	14.4***	20.0	17.8
42	23.2	19.5*	20.0			20.1	18.6
49	22.5*	20.0	19.5			20.2†	Died
56	21.5	18.6**	18.7**			18.2	42nd
63	20.5	18.2	19.2			16.8	day.
70	20.8**	19.3	20.0			15.9	
77	24.2	20.2	21.1			16.0***	
84	24.6	21.0***	21.7***			17.8	
91	24.5	17.5	17.6			17.0	
98	24.4***	20.3	Died			17.0	
105	22.0	20.0	97th				
112	23.0	19.3	day.				
119	23.6	20.0†					
126	Died	19.5					
133	119th	17.5					
140	day.	16.0‡					

* Increased sucrose to 10 per cent.

** Added cystine to the ration, 1 per cent.

*** Changed to "hydrolyzed casein preparation" made from casein digested for 3½ months and containing 24.25 per cent peptide nitrogen, negative biuret test.

† Changed to a "monoamino-acid mixture" plus cystine, in place of the hydrolyzed casein ration.

‡ Changed to "monoamino-acid mixture" plus the diamino-acids from gelatin and cystine. The animal refused food and was placed on the preliminary ration.

§ Added flowers of sulfur to the ration.

Mice 30 ♀ and 72 ♀, and then declined slowly, possibly indicating that the supply of this essential substance was slowly being depleted.

The fact that casein is low in cystine led to the suspicion that the deficiency might be due to the lack of this amino-acid; consequently about 1 per cent cystine was added to the ration fed to Mice 30 ♀, 71b ♀, and 72 ♀. A marked change was noticed immediately; Mouse 30 ♀ increased more than 3 gm. in weight within 1 week after the addition of cystine and main-

tained this level for 21 days. The other two mice, 71b ♀ and 72 ♀, increased from 18.6 and 18.7 to 21 and 21.7 gm. respectively, in the course of 28 days. A noticeable fact was that the mice were losing hair in the later stages on Ration 6, but this ceased as soon as cystine was added to the ration, and within a short time the mice had sleek coats again.

These data indicate rather clearly that cystine was the missing constituent of the hydrolyzed casein, and lend support to the conclusion of previous investigators that cystine must be regarded as an essential amino-acid, both for maintenance and growth.

Mice 30 ♀, 71b ♀, and 72 ♀ were then placed on a hydrolyzed casein ration prepared by digestion with pancreatin for 3½ months instead of 2 months. Mouse 72 ♀ died, after having been on the ration for 13 days. Mice 30 ♀ and 71b ♀ did not take to the food at first, but after a week they ate the ration fairly well. Mouse 30 ♀ maintained its weight for 3 weeks, when it died. Mouse 71b ♀, after maintaining its weight for 5 weeks, was changed to a ration made up as follows:

Ration 7.

	per cent
"Monoamino-acid mixture" prepared as described above	9
Cystine	1
Butter fat	10
Protein-free milk	28
Dextrin	42

A steady decline set in, although the food consumption was good. The mouse dropped in weight from 20.0 to 16 gm. in 3 weeks, indicating that the material removed by precipitation with phosphotungstic acid in acid solution is either wholly or in part necessary for the maintenance of adult mice. Further experiments will be reported later confirming this point.

The behavior of this mouse is interesting. It lived on hydrolyzed casein, containing only amino-acids and the simplest peptides as the sole source of nitrogen, for 140 days.

To ascertain whether the animal body is able to synthesize cystine when flowers of sulfur are added to a ration deficient in cystine, three mice, *i.e.*, 72b ♀, 71a ♀, and 77 ♀, after a rapid decline in weight on Ration 6, had flowers of sulfur added to their ration instead of cystine. The loss in weight continued, showing that the animal body is not able to utilize sulfur to synthesize cystine. These animals were then placed on a ration made from casein digested with pancreatin for 3½ months instead of 2 months; Mice 72b ♀ and 71a ♀ refused food and were placed on the preliminary

ration. Mouse 77 ♀ maintained itself for 3 weeks and was then changed to the monoamino-acid ration (Ration 7). On this ration the mouse rapidly declined in weight for 4 weeks. The monoamino-acid mixture was then substituted by hydrolyzed casein digested for $3\frac{1}{2}$ months. Loss in weight ceased, and the animal maintained its weight for 3 weeks.

The results secured with this animal also lend support to the conclusion regarding the indispensability of the diamino-acids for maintenance.

Mouse 35a ♀, after maintaining its weight for 5 weeks on Ration 6, died suddenly.

The greater nutritive efficiency of the casein hydrolyzed for $3\frac{1}{2}$ months as compared with that hydrolyzed for 2 months seems clear from the data of these experiments, though an explanation of this relation is difficult.

The food consumption of the experimental animals was quite satisfactory, and declines in weight cannot be attributed to too low a food intake. Each animal was offered daily about 2 gm. of food which was usually consumed, except where noted. Several other mice were placed on hydrolyzed casein, but refused food and consequently lost weight rapidly. These results are not reported here.

Experiment 7.—Another experiment was started in which the nitrogenous portion of the ration was made up of the monoamino-acid mixture from casein supplemented with the diamino-acids from gelatin plus cystine, histidine, and tryptophane.

The food mixture was made into a biscuit form from the following materials:

Ration 8.

	per cent
Monoamino-acids from casein	8.0
Diamino-acids from gelatin	1.5
Cystine	0.25
Histidine	0.25
Tryptophane	0.50
Sucrose	10.00
Butter fat	10.00
Protein-free milk	28.00
Dextrin	42.00

Four mice received this ration; three refused it, but the fourth, 90 ♀, ate it in sufficient quantities after 14 days, maintaining its weight from that time for 42 days. Below are given the changes in weight.

Days.	No. 90 ♀. *gm.*
1	27.5
7	24.5
14	22.0
21	20.2
28	21.0
35	20.7
42	20.8
49	20.8
56	23.2

This experiment, although carried out with only one mouse, tends to show that the ration fed is adequate, provided the animals take sufficient of it; furthermore, it helps to overthrow the objection which might be raised that the monoamino-acid mixture lacked some essential monoamino-acids other than tryptophane and that the losses in weight obtained in Experiment 6 might also be due to their absence. Here maintenance was secured with a ration composed of the monoamino-acid mixture, supplemented with the diamino-acids and tryptophane, showing that the loss in weight in the case of Mice 71b ♀ and 77 ♀ on the monoamino-acid mixture alone was due to the absence of the diamino-acids. Further proof of this will be given in the next experiments.

Experiment 8.—To improve the taste of the rations and increase their calorific value, in this and subsequent experiments, more butter fat and lard were added. Furthermore, instead of mixing all the ingredients together and rubbing them into a paste, the following procedure was adopted. The lard and butter fat were melted on the water bath, and the amino-acid mixture was stirred into the melt. The starch, protein-free milk, sucrose, and lactose, previously well mixed, were then added, and the whole mass was well stirred. By adopting this method, apparently, the food mixtures were made more palatable, and the mice took more of the food although some difficulty was experienced in a few cases. The following ingredients constituted the ration used in this experiment:

Ration 9.

	per cent
Monoamino-acid mixture from casein	11.5
Cystine	0.25
Tryptophane	0.25
Sucrose	12.00
Lactose	.00
Protein-free milk	28.00
Starch	14.00
Butter fat	18.00
Lard	10.00

Before being placed on this mixture, the animals used in this and subsequent experiments were fed for about 2 weeks on a ration composed of 33 per cent skim milk powder, 18 per cent butter fat, 10 per cent lard, and 39 per cent starch. Five mice were placed on Ration 9 with the following results.

Days.	No. 203a ♀.	No. 203 ♀.	No. 204a ♀.	No. 205a ♀.	No. 205 ♀.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
1	24.0	23.2	31.0	22.0	22.0
7	20.0	18.6	22.0	18.0	18.0
14	18.6	16.5*	20.0*	17.0*	17.5*
21	17.0*	16.7	18.0†	17.5	20.5
28	20.7	18.7		19.0	22.5
35	20.8	18.5		19.5	21.0
42	21.0	17.5†		18.5†	19.5†

* Changed to Ration 10: 9.25 per cent monoamino-acid mixture, 2 per cent diamino-acids from gelatin, 0.25 per cent cystine, and 0.5 per cent tryptophane.

† Refused food and placed on preliminary ration.

The animals were offered 2.5 gm. of food each day and took it satisfactorily, except where noted. It will be observed that there was a rather large decline in weight between the 1st and 7th days; thereafter the loss was gradual. Mice 203a ♀ and 205 ♀ made very pronounced gains when the diamino-acids were added. In the case of Mice 203 ♀ and 205a ♀ the gain in weight was smaller and more gradual. The experiment was discontinued after 42 days, because the animals were not taking the food well, and it was felt that the loss in weight of the experimental subjects was due to the fact that they were not consuming sufficient food to satisfy their energy requirements.

The results of this experiment, together with those of Experiments 6 and 7, point rather clearly to the conclusion that all or

some of the constituents of hydrolyzed casein removed by the precipitation with phosphotungstic acid in acid solution, *i.e.*, the diamino-acids, are essential for the maintenance of adult mice. The fact that a similar ration with only 2 per cent of the monoamino-acid mixture replaced by the diamino-acids from gelatin caused the mice to increase in weight and maintain themselves shows rather definitely that the loss in weight on the monoamino-acid food mixture was due to the absence of the diamino-acids, and not to the removal of some essential monoamino-acids which may have been in peptide combination with the diamino-acids.

Experiment 9.—Having shown that either all or some of the diamino-acids are necessary for the maintenance of adult mice, the next step was to ascertain which of the diamino-acids are essential. In Experiment 6 it was shown that cystine was necessary; hence in the present trial it was added to all the rations employed.

The rations of this experiment were similar to those used in Experiment 7, except that the nitrogenous portion was supplied by the monoamino-acid mixture plus cystine and tryptophane and one of the three diamino-acids, arginine, histidine, or lysine. Four mice were placed on a diet in which cystine and histidine were the only diamino-acids present. Two of the subjects refused the food and were taken off the experiment; the other two, 203a ♀ and 203 ♀, did well on the ration and maintained their weight for 28 days. The food mixture was made up as follows.

Ration 11.

	per cent
Monoamino-acid mixture from casein	10.75
Cystine	0.25
Tryptophane	0.25
Histidine	0.75
Sucrose	12.00
Lactose	6.00
Starch	14.00
Protein-free milk	28.00
Lard	10.00
Butter fat	18.00

After having maintained themselves on this ration for the period mentioned above, the histidine was replaced by an equal amount of arginine, and two more mice, 91a ♀ and 91 ♀, were placed on this second ration (Ration 12). The four animals maintained themselves satisfactorily

for 21 and 28 days, and thereafter the arginine was replaced by the same amount of lysine (Ration 13). Four other mice, 205a ♀, 205 ♀, 207a ♀, and 207 ♀, making a total of eight, were fed this ration, and all declined in weight. Mouse 207 ♀ lost weight much more rapidly than its mate. After it was on the lysine ration for 28 days and had declined from 20.3 to 13.0 gm. in weight, the lysine was replaced by arginine, resulting in an increase in weight to 14.5 gm. Thereafter the arginine was again replaced by lysine, when decline set in once more. The eight mice were then changed to a diet in which the lysine was replaced by histidine (Ration 11), but unfortunately the animals did not take the food very well. However, they consumed sufficient food to maintain their weight for 14 days.

The significant point about this part of the experiment is that as soon as histidine was added the mice ceased losing weight, and although they did not regain much weight, they nevertheless maintained themselves.

The animals were then divided into two lots of four each; Lot 1, made up of Mice 205a ♀, 205 ♀, 207a ♀, 207 ♀, were fed the "monoamino-acid mixture" prepared from the enzyme digest, supplemented with cystine, tryptophane, and histidine (Ration 11). These animals consumed sufficient of the food to maintain their weights fairly well for 14 days, but the food intake was not very satisfactory. The other lot of mice, 203a ♀, 203 ♀, 91a ♀, and 91 ♀ were offered a similar ration, except that the monoamino-acid mixture was prepared from an acid digest, and contained no peptides. This lot refused the food and, being in a low state of nutrition, two of the animals died (91a ♀ and 91 ♀); the other two were placed on the preliminary ration. The changes in weight of the mice in this experiment were as follows.

Days	Weight.		Food intake.	Weight.		Food intake.[5]	Weight.		Food intake.[5]	Weight.		Food intake.[5]
	No. 20[illegible] ♀.	No. 20[illegible]		No. 207a ♀.	No. 207 ♀.		No. 205a ♀.	No. 205 ♀.		No. 91a ♀.	No. 91 ♀.	
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	24.0[3]	22.0[3]		22.5[2]	20.3[2]		23.2[2]	20.8[2]		27.5[1]	23.5[1]	
7	22.5	17.0	24	22.5	16.5	20	19.5	18.2	25.5	21.0	20.0	18.0
14	22.3	17.5	32	21.7	15.0	18	19.5	17.0	17.5	21.5	20.3	17.5
21	22.1	15.0	34	20.5	13.0	17.5	20.8[9]	16.7[9]	42.5[7, 9]	22.5	20.0	22.5
28	21.7	16.0	34.5	21.0	13.0[1]	22.5	18.0	15.5	17.5	20.0[2]	20.1[2]	35.0[7]
35	22.5[1]	17.5[1]	32.5	20.0	13.7	17.5	17.5[3]	14.7[3]	19.0	18.5	16.0	23.0
42	22.5	17.5	25.5	19.0	14.5[2]	18.5	17.5	15.0	16.0	16.4[4]	15.5[4]	17.5
49	23.5	18.7	35.0	18.2	12.0	17.5	16.7	15.5	17.7			1.5[8]
56	23.7	18.8	35.0	18.0[3]	11.7[3]	18.5			1.58[8]			
63	23.5[2]	18.5[2]	35.0	18.0	11.7	25.0						
70	21.0	16.0	35.0	18.5	11.0	20.8						
77	18.5[4]	15.2[4]	23.6			1.35[8]						
84		14.0[3, 6]	23.5									
91		14.5	2.18[8]									

[1] Monoamino-acid mixture (enzyme digest) + cystine + tryptophane + arginine (Ration 12).

[2] Monoamino-acid mixture (enzyme digest) + cystine + tryptophane + lysine (Ration 13).

[3] Monoamino-acid mixture (enzyme digest) + cystine + tryptophane + histidine (Ration 11).

[4] Monoamino-acid mixture (acid digest) + cystine + tryptophane + histidine (Ration 14).

[5] Food intake per week for two mice.

[6] No accurate account kept of food intake.

[7] Scattered food badly.

[8] Average daily food per mouse.

[9] 9 day period.

From these data it is obvious that histidine and arginine are interchangeable and that either one of them in the presence of cystine is able to support maintenance for the periods given. Maintenance in the absence of both is not possible, thus confirming the work of Ackroyd and Hopkins,[5, 6] except that their experiments were conducted with growing rats and the ones under discussion with adult mice. Whether lysine is dispensable cannot be so definitely concluded from these trials, although the indications point strongly in that direction. Allowance must be made for

the possibility that there may be some lysine in the nitrogenous portion of the protein-free milk which was used in all the rations. The amount present at most must be very small, but even the presence of small quantities of an essential substance in a ration is sometimes quite significant. Should lysine not be necessary for maintenance, an excellent illustration would be on hand showing the qualitative difference between the requirements for growth and for maintenance; in the former case this amino-acid is essential. In this connection may be mentioned the work of Osborne and Mendel[27] with zein supplemented by tryptophane as the only source of nitrogen, except that contained in the protein-free milk. A rat maintained its weight on this ration for over 180 days. This finding is in harmony with the results reported here, as no investigator has as yet reported the presence of lysine in zein.

The behavior of Mice 203a ♀ and 203 ♀ is particularly interesting. These animals were started on a diet with histidine and cystine as the only diamino-acids and maintained their weights for 28 days; arginine was substituted for histidine and maintenance continued for 28 days more, but when the arginine was removed and replaced by lysine loss in weight ensued immediately.

SUMMARY.

1. Synthetic rations, adequate for the maintenance of adult mice, have been compounded with purified casein and with casein hydrolyzed with enzymes and containing only amino-acids and the simplest peptides, as indicated by a negative biuret test. Except for the presence of a small amount of nitrogen in the protein-free milk used, this hydrolyzed product formed the sole source of nitrogen in the food mixture.

2. Casein suspended in boiling water for 2 hours does not lose its nutritive value.

3. Heating casein for 1 hour in an autoclave at 15 pounds' pressure impairs or possibly destroys its nutritive value. This is in agreement with the findings of McCollum and Davis

4. If the diamino-acids are removed from hydrolyzed casein with phosphotungstic acid in acid solution, the residual amino-acids are inadequate for the maintenance of adult mice. This is not in agreement with the findings of Henriques and Hansen, but confirms those of Ackroyd and Hopkins.

5. Cystine appears to be necessary for the maintenance of adult mice.

6. Arginine and histidine seem to be interchangeable in nutrition. Full grown mice are able to hold their weight, when either one of them, together with cystine, is present in the ration. In the absence of both, loss of weight results. This is in harmony with the work of Ackroyd and Hopkins in the case of growing rats.

7. Lysine does not appear to be necessary for the maintenance of adult mice.

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STUDIES ON ENZYME ACTION.

XV. FACTORS INFLUENCING THE PROTEOLYTIC ACTIVITY OF PAPAIN.

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The plant protease, papain, has been the subject of numerous studies. The earlier literature on the subject has been reviewed in some detail by Mendel and Blood (1910), whose paper embodies the results of one of the few careful investigations of this ferment. The work of these authors is, however, almost entirely qualitative in character. In extending the general plan of enzyme study that has been undertaken in this laboratory, it seemed advisable to reinvestigate some of the facts concerning this enzyme, employing some of the more accurate methods that have been recently developed. This ferment lends itself very well to chemical study since it may be obtained in large amounts of fairly uniform activity.

In this paper some observations concerning the purification of the active material and a consideration of the influence of acidity and the quantitative relationship between enzyme and substrate will be presented. The peculiar behavior of HCN in accelerating the action of papain has been studied further.

Purification of Papain.

In view of the method by which commercial papain is produced,[1] it seemed desirable that a more refined material be used in this work, especially since some of the contaminating material might have a deleterious action on the enzyme. It also might be

[1] Pratt, D. S., *Philippine J. Sc.*, 1915, x, 1.

expected that a refined material would be more uniform in composition and would therefore make the different experiments more comparable.

The papain as obtained from Parke, Davis and Company was a light brown finely divided powder nearly all of which was soluble in water, giving a yellow to brown solution. The insoluble material settled rapidly and the solution could readily be removed from it by decantation. A few preliminary experiments were carried out with a view to determining to what extent purification of the active material could be accomplished.

15 gm. of papain were ground up with 500 cc. of distilled water and allowed to stand over night. The next morning a portion of the suspension was filtered through asbestos and treated as follows.

A. 50 cc. of the filtered solution were treated with 150 cc. of acetone and the mixture was allowed to stand 2 hours. The precipitate that formed was centrifuged off and the mother liquor decanted. The precipitate was then taken up in 100 cc. of water. This is referred to as Solution A.

B. 50 cc. of the filtered solution were added to 100 cc. of acetone and then treated as in A, the water solution of the resulting precipitate being Solution B.

C. 50 cc. of the filtered solution were added to 250 cc. of 95 per cent alcohol and then treated as in A, the water solution of the resulting precipitate being Solution C.

As controls, the original filtered and unfiltered solutions diluted with an equal volume of water were used. These solutions were D and E respectively.

To test the activity of the solutions 5 cc. were allowed to act on 25 cc. portions of 1 per cent gelatin containing 0.2 per cent of tricresol as a preservative. The solutions were incubated at 37° for 17 hours and the increase in "formol" titration over the blanks was used as a measure of the activity.

Solution.	Formol titration.			
	Substrate blank.	Ferment blank.	Ferment + substrate.	Action.
A	2.65	0.35	4.00	1.00
B	2.65	0.30	4.00	1.05
C	2.65	0.45	4.90	1.80
D	2.65	1.00	4.60	0.95
E	2.65	1.10	5.00	1.25

From the above experiment it is apparent that the active material may be concentrated by the precipitation with two to three volumes of acetone or with five volumes of 95 per cent alcohol; the latter procedure in addition to effecting a concentration of the active material seems to remove some of the inhibiting contaminants. It is also to be noted that the precipitation removes from the active material a relatively large amount of substance giving a formol titration. It is possible to fractionate commercial papain in this way so that about two-thirds of the material can be removed without appreciably impairing the activity of the remaining material.

Using the information obtained in the above experiments, about 50 gm. of papain were purified for use in the experiments given below.

150 gm. of commercial papain were rubbed with 4,500 cc. of water and allowed to stand over night. The next morning 4,000 cc. of clear solution were siphoned off and without filtering were poured into 8 liters of acetone. The precipitate was allowed to settle. After standing 4 hours the clear supernatant fluid was decanted off and the precipitate filtered and washed with acetone. The precipitate was finally drained on a large Buchner funnel and then rubbed up with 800 cc. of warm water, and the turbid brown solution allowed to stand 36 hours in a tall cylinder, a layer of toluene acting as a preservative. After standing, the clear supernatant liquid was siphoned off and poured into 4 liters of 95 per cent alcohol, and the precipitate filtered on a Buchner funnel. The filtration proceeded very slowly, taking 24 hours. The precipitate was rubbed with 95 per cent alcohol and then with ether, and dried after filtration in a current of air. The drying was rather unsatisfactory, the material becoming light brown in color. The activity of the material was, however, very high, so that it is certain that the ferment is fairly stable in aqueous solution and precipitated in the presence of acetone, alcohol, and ether. This observation is contradictory to some of the statements that appear in the literature regarding the deterioration of papain. Other experiments also confirmed the conclusion reached here. Papain allowed to stand over night at 37° seems to show little if any deterioration. Dialysis of the ferment in collodion bags results in a certain loss of activity, the bag contents becoming less active while the dialysate becomes slightly active, the sum of the two or the combined action of both being less than that of the untreated aqueous solution that stood under the same conditions. The deterioration is accelerated by dialyzing at 37°.

Optimal Hydrogen Ion Concentration for the Papain Action.

Inasmuch as most ferments seem to have a definite range of acidity or alkalinity in which they exhibit their maximal activity, it seemed strange that papain should, as stated in the literature, act equally well in acid or alkaline solution. To throw more light on this point a series of experiments was undertaken to determine at which hydrogen ion concentrations papain was most active proteolytically. The data recorded below are typical of the results obtained in different experiments so the conclusion seems justified that papain, in common with other ferments, has an optimal hydrogen ion concentration, in this case approximately 10^{-5} N. In all cases, the indicator method was used and the results are therefore not more accurate than a half a unit in the pH[2]. In the presence of proteins the indicator results are not entirely to be relied upon except for comparative purposes. The absolute hydrogen ion concentrations of the various solutions used cannot be given with certainty.

A 2 per cent solution of gelatin in water was treated with HCl and NaOH so that the solutions when tested with suitable indicators showed that they were of the hydrogen ion concentration desired. A 0.5 per cent solution of purified papain was divided into three parts and adjusted to 10^{-3}, 10^{-6}, and 10^{-9} N. 25 cc. portions of the various gelatin solutions were measured out and treated with 5 cc. of the papain solution of the same range of acidity. Duplicate blanks were set up with the papain and the gelatin and triplicate mixtures were made containing the protein and ferment.

Proteolytic Action of Papain at Various Hydrogen Ion Concentrations.

pH.		Action.
Initial.	Final.	
2	2	0.50
3	3.5	1.45
4	4	4.95
5	5	5.35
6	6	3.35
7	6	2.55
8	6.5	1.50
9	7	1.25

[2] The symbol pH is used interchangeably with the term hydrogen ion concentration and denotes numerically the negative exponent of 10.

Of the latter, one was used to test the hydrogen ion concentration before and after the flasks were allowed to stand in the incubator. The period of incubation was 22 hours. The results given in the column under actions are the formol titrations in cc. of 0.1 N alkali after correcting for all blanks.

The above experiment shows fairly conclusively that papain exhibits its greatest activity at an acidity equal to the concentration of the hydrogen ion of 10^{-5} N; *i.e.*, slightly more acid than is necessary to cause methyl red to change from yellow to red. The method of experimentation used above gives results which are entirely in accord with those obtained when the rate of cleavage of gelatin and egg white is followed at different hydrogen ion concentrations. It is interesting to note the changes in hydrogen ion concentration that occur during the proteolysis. In those cases either side of the optimum acidity, the tendency is for the solution to become more acid or alkaline, apparently tending to bring the solution to the optimum acidity. This is rather peculiar in view of the fact that at all times the solutions contain a large quantity of material that might act as buffer. In fact as the digestion proceeds the buffer action should become more marked since a greater number of amino and carboxyl groups are present. The only explanation that is apparent at present must involve an assumption that postulates two different types of cleavage products, depending on the hydrogen ion concentration. In one case we must assume the liberation of a preponderance of basic amino-acids or peptides, in the other an excess of acid compounds.

Having found that there was a definite hydrogen ion concentration at which papain was most active proteolytically, the question to what extent the ferment was decomposed on standing with acid and alkali was raised. To throw light on this point, papain solutions were treated with different strengths of acid and alkali and then neutralized to methyl red (hydrogen ion concentration 10^{-5} N) and allowed to act on gelatin. The actions were compared with those of the untreated solution of papain at the same hydrogen ion concentrations. Suitable blanks for enzyme and substrate were run and the results corrected for them. Toluene was used as a preservative.

Influence of Acids and Alkalies on the Proteolytic Activity of Papain.

Concentration.	Time of standing.	Action.	Remarks.
	hrs.		
0.5 N acid	4	0.10	Incubation with gelatin 41 hrs.
0.1 " "	4	0.25	
Water	4	4.95	
0.1 N alkali	4	0.20	
0.5 " "	4	0.05	
0.05 N acid	1	2.30	Incubation with gelatin 18 hrs.
0.02 " "	1	2.90	
Water	1	4.10	
0.02 N alkali	1	3.65	
0.05 " "	1	2.85	

The above experiment shows that the ferment is sensitive to both acid and alkali, the latter being less destructive.

Quantitative Relationships between Papain and Its Substrate.

In studying the changes that occur when an enzyme and its substrate react it is evident that while the enzyme is affecting the substrate, the latter is modifying the activity of the enzyme. It has been stated in the literature of the subject that the proteolytic activity of enzymes follows the simple mass action law, this conclusion being deduced from the study of the kinetics of hydrolysis. There are several reasons why it is perhaps a fruitless task to try to formulate a statement of the kinetics of the reaction involving the enzymatic cleavage of a protein. First, the system involves two colloidal components and it is therefore unlikely that solution kinetics will apply, but instead adsorption phenomena may be the basic factors (Nelson and Vosburgh, 1917). Second, the cleavage of protein does not represent a reaction where one stage is completed before another begins but rather a complex of a number of simultaneous reactions.

In order to determine what rôle the relative quantities of enzyme and substrate play in the action of papain on protein the following experiment was carried out.

A series of solutions of gelatin of definite concentration were treated with acid until their hydrogen ion concentration as indicated colorimetric-

ally was 10^{-5} N. Similarly a series of papain solutions were prepared. Mixtures of these as indicated in the table were incubated at 37° for 24 hours and the extent of cleavage was determined by the formol titration. Toluene was added as a preservative. All the data recorded are corrected for suitable blanks on enzyme and substrate.

Proteolysis with Varying Concentrations of Enzyme and Substrate.

Papain.	Gelatin.			
	mg. 125	*mg.* 250	*mg.* 500	*mg.* 750
	Formol titration.			
mg. 5	1.70	2.65	3.25	3.55
10	2.10	3.50	5.05	5.90
25	2.25	4.40	7.30	8.95
50	2.45	4.70	8.55	11.00

The results of the above experiment lend support to the view that in the cleavage of protein by papain there is a two stage reaction, the first involving a combination of enzyme and substrate, and the second the cleavage of this intermediate compound to give the enzyme and the split products of the protein. It will be seen from the curves that when the amount of the substrate present is relatively small, the proteolysis is not proportional to the enzyme concentration but tends to a definite point, the addition of further enzyme producing little additional cleavage. In the case where the ferment is kept constant, the proteolysis depends on the ratio of substrate to ferment. If the ferment concentration is large the "formol" titration after proteolysis is almost directly proportional to the quantity of substrate. With smaller substrate concentrations the action is dependent on the concentration up to a certain point, after which the addition of more substrate causes little more action. These findings would indicate that a given quantity of enzyme can handle a given quantity of substrate after which the addition of either component leads to no further action. This brings the action of papain into the same class as urease, invertase, and lipase. In considering the relations of enzyme and substrate, it appeared of interest to determine to what extent the addition of more enzyme

and substrate to a digestion mixture would affect the results. The results of the experiments are summarized in the following table. In the table indications are made of the amounts of ferment and substrate added on the 1st and 2nd day and the "formol" titrations of the resulting digestion mixture at the end of the 2nd day recorded under Action, the figure given being corrected for all blanks.

Influence of the Addition of Ferment and Substrate to a Digestion Mixture.

No.	1 per cent papain solution.		5 per cent gelatin solution.		Action.	Remarks.
	1st day.	2nd day.	1st day.	2nd day.		
	cc.	*cc.*	*cc.*	*cc.*		
1	5	0	5	0	2.40	
2	5	5	5	0	2.55	Slight increase due to additional quantity of ferment.
3	10	0	5	0	2.70	Twice the original quantity of ferment gives only slightly larger action than No. 2.
4	5	0	5	5	3.50	Addition of more substrate shows that active ferment remains but that action on 2nd day is smaller than on 1st.
5	5	0	10	0	5.30	Twice the original quantity of substrate gives much larger action than No. 4. Time factor may play a rôle.
6	5	5	5	5	4.35	Two individual additions of enzyme and substrate do not cause twice the action in No. 1, due possibly to a retarding effect of the products.

These experiments also support the view that there is a definite quantity of ferment required for a given amount of substrate and that an excess causes little more action.

The striking action of HCN on proteolytic activity of papain has been noted by Vines (1903) and Mendel and Blood (1910). The experiments of these authors were for the most part qualitative in character and served to show that HCN had a definite rôle as a specific activator of papain action, altering the character of the reaction to such an extent that more extensive cleav-

Proteolytic Activity of Papain-HCN at Varying Hydrogen Ion Concentrations with Varying Concentrations of Papain.

Experiment 1. Gelatin...... 500 mg. HCN ...... 53 " Papain...... 2.5 " Volume...... 35 cc.			Experiment 2. Gelatin .. 500 mg. HCN...... 53 " Papain .... 5 " Volume .... 35 cc.			Experiment 3. Gelatin.... 500 mg. HCN ..... 67 " Papain.. 12.5 " Volume.... 35 cc.			Experiment 4. Gelatin..... 500 mg. HCN........ 53 " Papain ...... 25 " Volume.. .... 35 cc.		
pH.		Action.	pH.		Action.	pH.		Action.	pH.		Action.
Initial.	Final.		Initial.	Final.		Initial.	Final.		Initial.	Final.	
						2.5	3.5	0.55	2	2	0.50
3	3.5	0.40	3	3.5	1.50				3	3.5	1.90
4	4.5	3.10	4	4.5	6.00	4	4	9.30	4	4	10.45
5	5	3.90	5	5	6.00	5	5	8.70	5	5	9.70
6	5.5	4.85	6	5.5	6.05	6	6	8.50	6	6	9.75
7	5.5	4.90	7	5.5	6.25				7	6	9.40
						8	7.5	7.70	7.5	6	8.95
									8.5	6.5	8.80

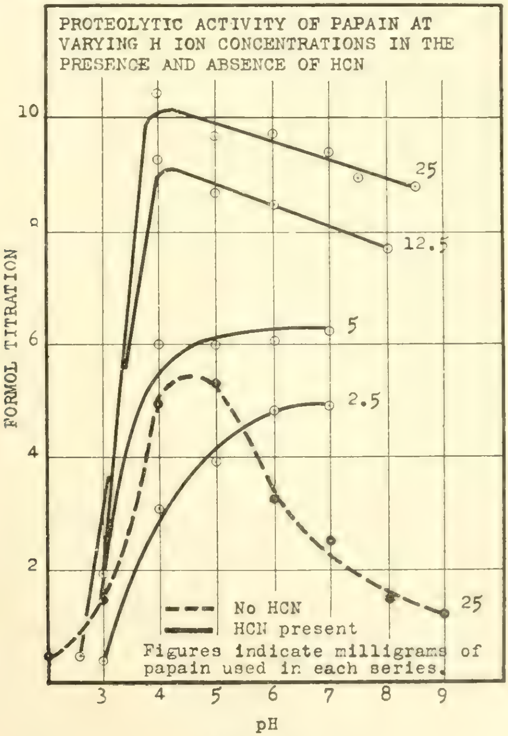

CHART 1.

age of the protein resulted. In considering the nature of the active groupings in papain it seemed desirable to review some of the experiments of the earlier workers, using more modern methods and taking into consideration the factors of acidity and the quantitative relationships between the various components of the reacting system.

The optimal hydrogen ion concentration for papain activity in the presence of HCN was determined in the same way as in the case where no HCN was used, the same ferment solution being taken, under much the same conditions.

The data above are in marked contrast to those obtained where no HCN was present. Instead of finding a definite hydrogen ion optimum for papain-HCN proteolysis we find that the ferment is equally active, or nearly so, over a wide range of acidity. The results obtained with lower concentrations of ferment leave the whole matter unsettled. Further work on this point involving more careful measurements of the hydrogen ion concentration by means of the gas chain method is planned. The rôle of the HCN is not at all clear. From experiments presented below, it would appear that the HCN combines with the papain and the substrate to form an intermediate compound which then undergoes cleavage much as the intermediary compound of papain and protein does. Under such circumstances it may be that the ternary HCN-papain-protein compound has different stability.

To determine whether proteolysis in the system papain-protein-HCN follows the same general scheme as in the system papain-protein, the following set of experiments were carried out.

Proteolysis with Varying Concentrations of Enzyme and Substrate. HCN Constant.

Papain.	Gelatin.			
	mg. 125	*mg.* 250	*mg.* 500	*mg.* 750
mg.	Formol titration.			
5	2.55	4.50	7.50	9.60
10	2.75	4.95	8.80	12.10
25	3.10	5.75	10.45	14.45
50	3.35	6.25	11.45	16.25

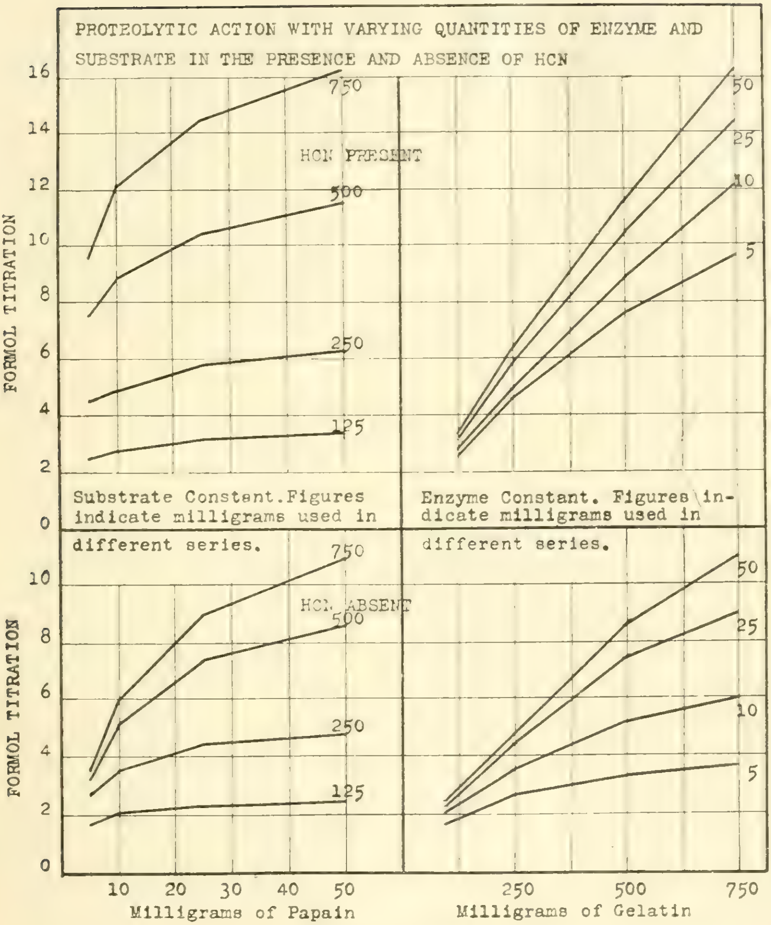

CHART 2.

The general plan was the same as before. The solutions of gelatin and papain were adjusted to pH 5 and the requisite amounts added to each experiment. The HCN solution used was prepared by dissolving 13 gm. of Kahlbaum's KCN in normal HCl, enough (200 cc.) of the latter being used to make the solution just neutral to methyl red. 2 cc. of this solution were used in each experiment. The volume of the digestion mixtures was 32 cc. Toluene was added as an additional preservative. The results given are corrected formol titrations.

To determine the relation between the extent of proteolysis and the amount of HCN used, the following experiment was

carried out. The same enzyme, substrate, and HCN solution were used as before, the quantities of the first two being kept constant while the third was varied. The results are given below.

Proteolysis with Varying Quantities of HCN, Enzyme, and Substrate Constant. Papain 10 Mg., Gelatin 750 Mg., Volume 30 Cc.

HCN.	Formol titrations.		
	Titration.	Action due to HCN.	Action per unit HCN.
cc.			
0	5.30		
1	10.75	5.45	5.45
2	12.10	6.80	3.40
5	12.80	7.50	1.50
10	13.90	8.60	0.86

The data presented in the two tables above indicate that proteolysis in the system papain-HCN-protein follows the same general laws as were noted above for the papain-protein system. Fixing two components we find that increase of the third tends to increase the total cleavage but not in proportion to the amount added. In fact there seems to be a tendency towards a definite maximum. The only explanation of this phenomenon that is apparent is one which assumes the existence of an intermediary ternary compound in which all three components are present in definite ratio. Any excess of enzyme or HCN over that necessary to give the proper combination seems to remain in the system without taking part in the reaction. If an excess of substrate be present, it would seem as though some of the material were awaiting its turn to be used.

In the experiments of Mendel and Blood various attempts were made to explain the action of HCN in papain proteolysis. They found that among other things methyl cyanide did not have the same effect as HCN, indicating that it was not the nitrile group that was involved. They noted that KCN had less action than HCN but this is undoubtedly due to the fact that the alkalinity of the KCN gave rise to an unfavorable hydrogen ion concentration. The only other substance that was found to be effective in accelerating the action of papain on protein was hydrogen sulfide. This led them to suggest that possibly the reducing properties of the two substances were responsible for their action.

If the reducing properties of HCN were responsible for its activity in papain proteolysis it might be expected that some of the HCN would be destroyed in the course of the digestion. The following experiment was carried out to test this point.

5 gm. of gelatin were dissolved in 200 cc. of water containing 0.4 per cent of tricresol. The solution was divided into two equal parts and 10 cc. of 1 per cent HCN solution added to each. To one 50 mg. of papain were added, and the other was used as a control. Both flasks were incubated at 37° for 24 hours, and then acidified with 15 cc. of 15 per cent sulfuric acid and distilled in steam, the distillates being collected in alkaline solution. The distillates were titrated with silver nitrate according to Liebig's method for cyanide determination with the following results:

	$AgNO_3$ cc.
10 cc. HCN solution	19.35
Control gelatin HCN	18.1
Papain HCN digestion	19 2

These results show clearly that HCN is not oxidized or converted into a compound that is not readily hydrolyzed by dilute acid. It is of course quite possible that the HCN enters into some combination that is not very stable in the presence of acid and can therefore be recovered completely with the method used. Further experiments on this point are in progress.

It has been claimed that in the course of papain proteolysis free amino-acids are liberated. Mendel and Blood obtained evidence of the formation of tryptophane in papain-HCN digestion. Abderhalden and Teruuchi (1906) claimed that the ferment could effect the cleavage of glycyltyrosine. In our experiments we have not found it possible to hydrolyze glycylglycine, alanylglycine, glycylalanine, alanylalanine, or glycyltyrosine with papain either in the presence or absence of HCN. In the experiments on alanylglycine the hydrogen ion concentration was varied over a wide range with no change in the result. How far up in the scale it is necessary to go to effect cleavage with papain is as yet unknown. Some experiments undertaken from a different point of view may be of interest in this connection.

200 cc. portions of 1 per cent gelatin and dried egg albumin solutions were adjusted to pH 5 and treated with 75 mg. of papain. The solutions were incubated at 37° and 25 cc. portions were withdrawn at the intervals

noted and titrated by the formol method. The results given under Action are the formol titrations corrected for the titration of 25 cc. immediately on mixing the ferment with the substrate. When the solutions were nearly in equilibrium, several 25 cc. portions were withdrawn and treated with HCN. The formol titrations were then made after incubation for the stated period.

Gelatin.		Egg white.	
Time.	Action.	Time.	Action.
hrs.		*hrs.*	
$1\frac{1}{2}$	0.35	3	0.35
3	0.65	5	0.35
$4\frac{1}{2}$	0.80	22	0.75
23	2.00	46	1.15
69	2.80	70	1.30
HCN added.			
72	3.15	94	3.30
96	3.95		

These experiments indicate that HCN is effective in renewing the proteolytic activity of papain even after equilibrium is apparently reached. Whether this effect is due to a cleavage of compounds of lower molecular weight or of some unattacked protein is as yet unsettled. *A priori*, it would appear that the former view is correct. It is the plan of the author to investigate further this whole question of HCN action in certain fermentations since it offers a new point of attack in the study of the chemistry of these reactions.

SUMMARY.

A method of purification of crude papain is presented.

The conditions of acidity for the optimum action of papain are found to be pH = 10^{-5}.

A consideration of the quantitative relations between papain and its substrate leads to the view that this ferment acts like urease, invertase, and lipase in forming an intermediary compound which is broken up into the cleavage products and liberates the enzyme.

Investigation of the action of HCN in papain hydrolysis leaves this question still unsettled. There seems to be some difficulty in defining a hydrogen ion optimum for papain-HCN proteolysis. The quantitative relations of the enzyme, HCN, and protein lend support to the view that there is a ternary intermediary compound formed by the components which then breaks down into cleavage products of the protein, enzyme, and HCN.

It has been shown that HCN may be recovered almost quantitatively from digestion mixtures, indicating that it is not utilized in the reaction of fermentation.

Papain, with or without HCN, seems to have no proteolytic effect on the dipeptides studied. HCN can renew proteolysis in papain digests that are almost in equilibrium.

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APPLICATIONS OF GAS ANALYSIS.

I. THE DETERMINATION OF CO_2 IN ALVEOLAR AIR AND BLOOD, AND THE CO_2 COMBINING POWER OF PLASMA, AND OF WHOLE BLOOD.

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In physics and in chemistry a multiplicity of methods of preparation terminate in a very few standard methods of measurement. Nothing in biochemistry corresponds to the place which the galvanometer and the chemical balance occupy in their sciences. On the contrary in biochemistry the methods of measurement are nearly as varied as those of preparation. As every method must be learned separately, the difficulties encountered by an investigator wishing to pass from one field of biochemistry to another are thus greatly increased.

We believe that gas analysis should become one of the standard methods of measurement in biochemistry. At present most workers in this field make no use of gas analysis. Those who wish to determine the alveolar air use a Fridericia (1) apparatus or Marriott's (2) color tubes; to determine the CO_2 combining power of the blood, the Van Slyke (3) mercury pump; for oxygen in blood the Barcroft (4) technique; for the study of the total respiratory exchange of man or animals, and for indirect calorimetry, the elaborate apparatus of Benedict. No two of these methods employ the same technique.

The object of each is, however, as we believe, more easily and more accurately accomplished by means of methods terminating in gas analysis along standard and long tried chemical lines. One who learns to do gas analysis for any one purpose has the equivalent of all of these methods at his command. While the more refined forms of gas methods involve skill and experience, such refinements are needed only when extreme accuracy is desired

and are for the most part quite unnecessary. A great deal can be done with sufficient accuracy with simple apparatus and technique.

An apparatus with which one may determine the CO_2 content of the alveolar air and the CO_2 content and combining power of

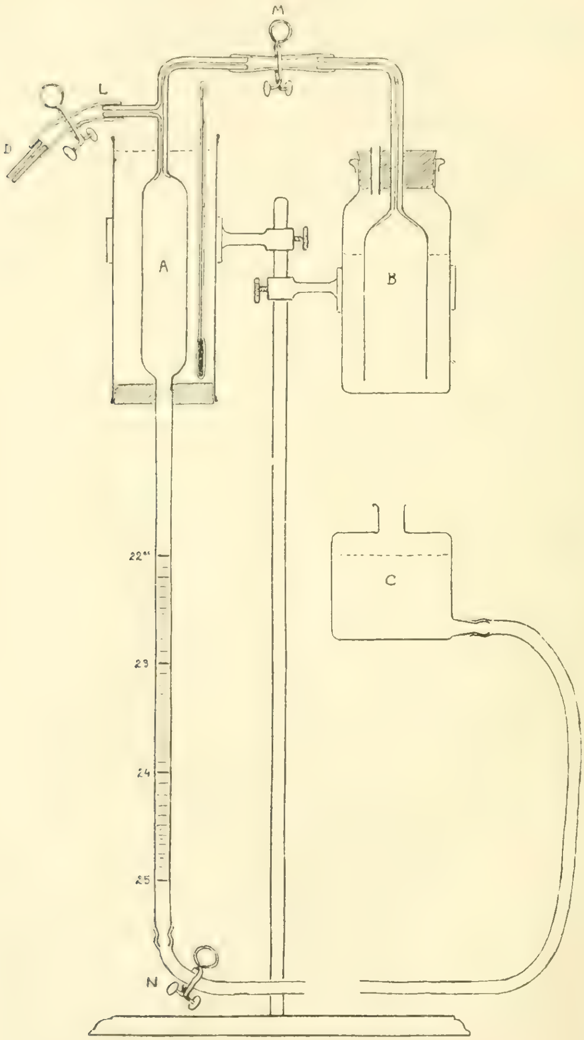

FIG. 1.

plasma or of whole blood is shown in Fig 1.[1] It involves practically nothing new or which has not been used in equivalent form by a long line of investigators. It consists of a 25 cc. gas burette (A) with a bulb containing about 21 cc., and a tube below graduated from 22 to 25 cc. in 0.02 cc., so that it can be read easily to 0.01 cc. Around the bulb of the burette is a jacket which is filled with water at room temperature, and a thermometer is placed in this fluid. At the top of the burette is a T-tube, one limb of which is connected by a rubber tube to a simple absorber (B) in which 10 per cent sodium hydroxide is placed. The compensating bottle (C) connected with the lower end of the burette by a rubber tube is filled with 1 per cent sulfuric or other acid. Spring clips or screw cocks (or hemostats) are placed at the points marked L, M, and N.

CO_2 Content of Alveolar Air.

The bladder of a football or other form of rubber bag is used as in the Higgins-Plesch (5) method of obtaining the alveolar air; the subject breathes into the bag four or five times and a spring clip is placed on its tube.

To prepare the apparatus for an analysis the leveling bottle is lifted until the bulb of the burette is full. The nipple (D) is put on at L and closed. The bottle is lowered while the clip at M is opened until the NaOH solution in the absorber is drawn up to a mark on the capillary glass tube above it. The clip at M is then closed, and that at L is opened. The bottle (C) is lifted until the burette is full, and a few drops of the fluid are run out (at L), the clip at N is closed, and the nipple, spring clip, and rubber tube, D-L, are taken off. The apparatus is now ready.

The tube of the bag holding the air to be analyzed is connected (at L) and the clip on the tube of the bag and that at the lower end of the burette (N) are opened. As the fluid falls in the burette it draws from the bag a sample of air, the amount being determined by the height at which the leveling bottle is held. Between 24 and 25 cc. of air are taken; the clip on the tube of the bag (at L) is closed. 2 or 3 minutes are allowed for the sides of the burette to drain, then the bottle is held so that the surface of the fluid in it is at the same level as that in the tube of the burette and the volume of gas in the burette is read to 0.01 cc. The clip on the absorber (at M) is now opened. The bottle is raised and lowered four or five times

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[1] It can be obtained from the Emil Greiner Company, 55 Fulton Street, New York.

so that the gas in the burette is driven over into the absorber and drawn back. The CO_2 in the sample of air is thus removed. The bottle is now lowered until the sodium hydroxide solution is drawn up into the capillary to the mark at which it stood at the beginning of the analysis, and the clip on the absorber (at M) is closed. After waiting the same length of time as previously for the sides of the burette to drain, the bottle is held so that the surface of the fluid in it is at the level of that in the burette and the latter is read off. The difference between the first and second readings divided by the first gives the percentage of CO_2. To turn this percentage into the partial pressure of CO_2 in mm. of mercury it is multiplied by a figure 40 mm. less than the prevailing barometric pressure; *e.g.*, 5.5. per cent CO_2 or $0.055 \times (760-40)$. This 40 mm. is the tension of water vapor in the lungs at body temperature. As regards the barometric pressure, it is sufficient for clinical purposes to use the mean pressure of the locality, neglecting the daily variations.

Duplicate analyses by this method should not differ by more than 0.02 cc. of CO_2, or 0.1 per cent, a sufficient degree of precision for all clinical and most scientific purposes.

A Modification of the Haldane-Priestley Technique.

The foregoing account starts with the Higgins-Plesch technique for obtaining alveolar air. For clinical purposes this is the easiest and yet a sufficiently close method. It is generally recognized, however, that the figures so obtained tend to be too high, approximating the gas tension of the venous rather than that of the arterial blood. The method originally employed by Haldane and Priestley—the single deep expiration through a wide tube and analysis of the last part of the expiration—although still the most accurate available, is difficult to apply on untrained or nervous subjects. In teaching elementary students one of us has observed that the chief trouble consists in the fact that the subjects are inclined to draw a deep inspiration before making the deep expiration through the tube. Of course this deep inspiration dilutes the pulmonary air with an excessive amount of fresh air and renders the CO_2 content too low.

This difficulty may be avoided by means of the apparatus shown in Fig. 2. It consists of a vertical tube of a capacity of 50 to 100 cc. held by a cork (cut as shown in the figure) in a wide mouthed bottle containing acidulated water. The subject is instructed to suck the water quickly up the tube until a bubble of air passes in, and then to make the deepest possible expiration

through the apparatus, and to close the clip. The apparatus is then connected with the burette and a sample of air analyzed. When one is about to exert a suction he has no inclination to make a preliminary deep inspiration as he does when the first conscious effort is to be expiratory.

In our opinion methods of calculating the alveolar CO_2 by formulas (6) involving a figure for (or including) the dead space are invalid, for the reason that, as shown by Henderson, Chillingworth, and Whitney (7), confirmed by Haldane (8), and conceded now by Krogh and Lindhard (9), the dead space is a very variable and only roughly measurable quantity, which in reality can itself be estimated only by using some figure for the composition of the alveolar air.

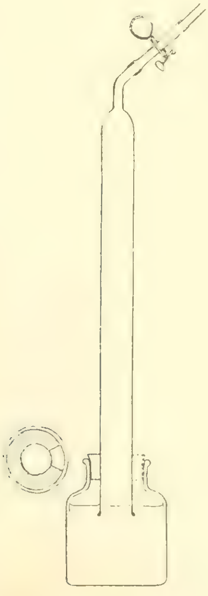

FIG. 2.

FIG. 3.

Determination of CO_2 in Blood.

The blood is drawn directly into an all glass syringe containing a glass bead and a small but known volume of ammonium or sodium or potassium oxalate, or the blood is drawn first and the oxalate afterward. The blood and oxalate are mixed by inverting the syringe a few times. The blood may also be run directly from an artery into a test-tube under a layer of petroleum oil and then either defibrinated (by stirring under the oil) or treated with a few grains of powdered oxalate. Excess of oxalate is to be avoided, as it interferes with laking the blood later on. For the blood gas analysis a tube (which may be called the diffusion tube) such as is shown in Fig. 3 is used. When a rubber stopper is inserted in its large end it should have a capacity between 27.5 and 28.0 cc. A bit of rubber tubing 4 or 5 cm. long is put on the capillary end and closed with a spring clip, or better with a bead valve consisting of a lead shot or piece of glass rod 5 to 8 mm. long inserted in the rubber tube. To be gas-tight the bead must be large enough to stretch the rubber considerably. Such a valve is opened by pinching the rubber tube over the bead with the fingers. A solution of 1 cc. of concentrated ammonia in 500 cc. of distilled water is used, and another of 20 per cent tartaric acid.

For an analysis 2 cc. of the dilute ammonia are put into the diffusion tube and 1 cc. of the blood is delivered with a pipette under the ammonia. The blood and ammonia are mixed and the blood laked by gently rotating the tube while held vertically. Then 0.5 cc. of tartaric acid solution is delivered with a long pipette below the laked blood. A 0.5 cc. pipette of sufficient accuracy is easily made of a piece of common glass tubing by drawing this volume from a burette and marking with a file—half a drop more or less is unimportant.

A rubber stopper is now inserted in the large end of the tube and the tube is laid horizontally and rolled rapidly for 3 minutes. The acidified blood spreads in a thin film which is continually renewed by the rotation and allows rapid diffusion of the liberated CO_2 into the air in the tube. For the rolling it is convenient to lay the tube in a rack such as shown in Fig. 4 and to connect it to a slow running motor by means of a belt. If the motor is not available the tube may be laid on a sheet of blotting paper or strip of cork and rolled back and forth by means of a strip of wood covered with blotting paper or cork (to avoid the heat of the hand). The essentials of this rolling are that rapid diffusion be obtained from the blood to the air in the tube without the production of any bubbles or foam.

The analyzer is now arranged for a gas analysis exactly as above described for alveolar air; that is, the bulb is filled with fluid and pinch-cocks are closed. The small end of the blood gas tube is attached at L, while the lower end dips into a beaker of slightly acidulated water (see Fig. 5). The rubber stopper is now withdrawn under water, the pinch-cocks at L and N are opened, and by lowering the leveling bottle all the air is drawn from the blood gas tube into the burette. The pinch-cock or bead valve at L is closed and the burette is read. The air is passed into the absorber

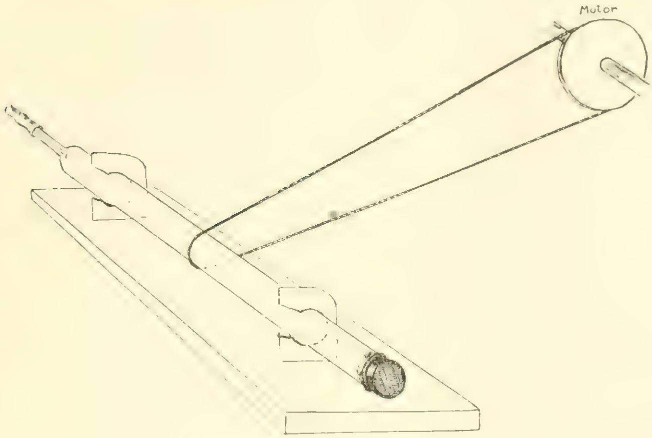

Fig. 4.

five times and the volume again read exactly as previously described. The decrease of the volume (not the percentage decrease) is the volume of CO_2 which has come from the blood. Every 0.01 cc. represents (with corrections) one volume per cent CO_2 in the blood.

A correction must be made for the CO_2 which remains in solution. At temperatures of 14–18° the solubility is about 1.0, 18–22° about 0.9, and 22–26° about 0.8. To make this correction it is usually sufficient merely to add one-tenth to the CO_2 found in the analysis. This quantity is estimated by multiplying the CO_2 found in the analysis by a fraction of which the numerator is the volume of acidulated blood and the denominator the volume of air in the tube, and then multiplying again by the coefficient of solubility at the temperature of the analysis.

An occasional analysis should be made without blood to determine the CO_2 which the ammonia solution may hold, and this correction is subtracted from each blood analysis. The ammonia should of course be kept tightly stoppered as it accumulates CO_2 from atmospheric air if exposed.

Duplicate analyses should agree to within 0.03 cc. of CO_2 in 1 cc. of blood.

This gives the CO_2 at the prevailing temperature and pressure. The correction to 0° and 760 mm. barometer may be obtained

from the table in a chemical calendar or calculated by multiplying the volume of CO_2 at the prevailing temperature and pressure by the fraction $\frac{273 \times 760}{(\text{temperature} + 273) \times \text{barometer}}$.

CO_2 Combining Power of Plasma.

Blood obtained as previously described is centrifuged. The plasma is brought into gaseous equilibrium with alveolar air in the following way (virtually that of Van Slyke). The last half of a normal expiration is expelled through a wash bottle or large test-tube containing glass beads to absorb the moisture into a 250 cc. separatory funnel containing about 3 cc. of plasma. The funnel is immersed in a water bath at body temperature and spun in such a way as to spread the plasma in a thin film over the glass surface. After spinning for about a minute another portion of alveolar air is introduced into the funnel and the spinning repeated. It is well

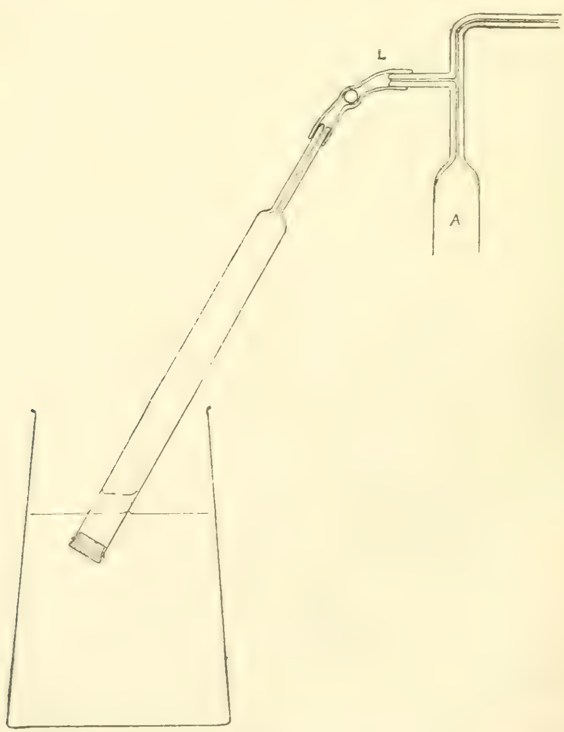

Fig. 5.

for beginners in this technique to make sure that the alveolar air used to obtain this equilibrium contains the correct percentage of CO_2 (about 5.5), by attaching the rubber bag to the opposite end of the funnel and analyzing a sample of the air which is blown through the funnel into the bag.

An even better technique is to collect alveolar air in the rubber bag, to analyze it, and then to squeeze part of this air through the separatory funnel in which the plasma has been placed. The separatory funnel is placed in a water bath and spun and the process repeated as above described.

After the plasma has been brought into equilibrium with the alveolar air 1 cc. is drawn into a pipette and placed in the blood gas diffusion tube under 2 cc. of dilute ammonia solution and mixed. Acid is added and the analysis for CO_2 made as previously described for blood.

TABLE I.

Comparative Results by Van Slyke Method and by Gas Analysis (Both Calculated by Means of the Van Slyke Table).

Case.	CO_2 in air used for saturation.	CO_2 in 1 cc. of plasma.		Difference by two methods.	Vol. CO_2 in 100 cc. of plasma at 0°, 760 mm.	
		Van Slyke method.	Gas analysis.		Van Slyke method.	Gas analysis.
	per cent	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Incomplete abortion.	5.84	0.67	0.66	−0.01	56	55
"	5.8	0.56	0.58	+0.02	45	47
Threatened abortion.	5.9	0.69	0.68	−0.01	57	56
After dilation and curettage.	5.4	0.59	0.64	+0.05	47	52
Toxemia of pregnancy.............	5.6	0.56	0.59	+0.03	45	48
Before operation for prolapsus...........	6.0	0.72	0.68	−0.04	60	56
Operation for prolapsus.................	6.2	0.69	0.71	+0.02	58	58
Toxemia of pregnancy.............	5.4	0.52	0.54	+0.02	40	42
"	5.7	0.56	0.53	−0.03	45	42
Menorrhagia.........	6.2	0.66	0.66	0.00	54	54
Eclampsia.						
Maternal blood....	6.2	0.32	0.34	+0.02	22	23
Fetal "	5.6	0.31	0.28	−0.03	21	18
Menorrhagia.........	6.7	0.58	0.60	+0.02	47	48
Normal labor.						
Maternal...........		0.39	0.43	+0.04	29	35
Fetal..............		0.48	0.55	+0.07	37	44
Eclampsia...........		0.37	0.36	−0.01	27	26

The degree of precision is about the same as with the Van Slyke apparatus. A careful determination is correct within 0.05 cc. of CO_2 in 1 cc. of blood. It is meaningless and absurd to express the results of such methods in figures carried out to tenths (or even hundredths) of 1 per cent, as some writers do. Comparative determinations by the Van Slyke method and by gas analysis are given in Table I. Duplicates by either method alone do not agree more closely.

CO_2 Combining Power of Whole Blood.

This is determined in exactly the same way as with plasma. As the figures determined by Van Slyke for the combining power of plasma and by Christiansen, Douglas, and Haldane (10) for whole blood are nearly identical, it appears that normal blood takes up (in 1 cc.) nearly the same amount as (1 cc. of) plasma. In a few comparative observations on animals we have found this to be the case, or that the whole blood takes up slightly more than an equal volume of plasma. From a few observations on patients it appears that possibly in eclampsia the combining power of the whole blood may be reduced much less than that of the plasma, suggesting the possibility of an actual increase in the CO_2 combining power of the corpuscles in this condition. Further observations however are needed on this point.

TABLE II.

Comparison of CO_2 Combining Power of Whole Blood and Plasma (Figures Corrected to 0° and 760 Mm.).

Case.	Plasma.		Whole blood, gas analysis.
	Van Slyke method.	Gas analysis.	
	vol. per cent	*vol. per cent*	*vol. per cent*
Dog, 2 hours' ether	30		31
Perineorrhaphy	58		60
4 months' pregnancy	51	53	73
Eclampsia	27	26	51

Other applications of gas analysis will be published in later papers of this series, including a refinement of the method above described for more precise determination of CO_2 and also for oxygen in blood.

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THE "VITAMINE" HYPOTHESIS AND DEFICIENCY DISEASES.*

A STUDY OF EXPERIMENTAL SCURVY.

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An elaborate experimental inquiry into the cause of the failure of rats to grow or to maintain life long when restricted to a diet of purified proteins, carbohydrates, fats, and inorganic salts led McCollum and Davis[1] to the conclusion that there were lacking in such food mixtures two substances or groups of substances, the chemical natures of which are still unknown, and which must be regarded as dietary essentials. McCollum and Kennedy,[2] after pointing out several reasons why the term "vitamine," introduced by Funk,[3] was unsatisfactory as a name by which to designate them, proposed the provisional terms "fat-soluble A" and "water-soluble B." The first is soluble in fats and is found in abundance in butter fat, egg fat, the ether extract from kidney, and also in considerable amounts in the leaves of plants, but in general in amounts too small in the seeds to supply the need of a growing animal. The second, which is soluble in water and in alcohol, has been shown to be present in abundance in wheat,[4] wheat germ,[5] maize,[6] alfalfa leaves,[7] and cabbage as well as in several foods of animal origin.

Funk[8] postulated the existence of a number of chemical substances essential in the diet, the absence of a single one of which would cause abnormal metabolism, in one case polyneuritis, another scurvy, another pellagra, and another rickets. He assumes that still other unidentified substances may be essential to growth. We recognized the possibility that our extracts which

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furnished the water-soluble B, and which in all cases have been found capable of inducing a cure of polyneuritic pigeons, the disease being induced by feeding polished rice, might contain this entire list of Funk's protective substances. We therefore instituted investigations designed to show whether the water-soluble B contained but one or more than one essential chemical constituent.

As a working basis the assumption seemed warranted that all animals as highly developed as the mammalia should require the same chemical complexes in the diet if growth and well-being are to be maintained. Experiments carried out with each of the natural foods enumerated above as supplying the water-soluble B showed that any one of these, when properly supplemented with inorganic salts, purified protein, and fat-soluble A (as butter fat), served to support growth.

This indicated the presence in each of these foods of all unidentified water-soluble dietary essentials necessary for growth and prolonged well-being. We were mindful of the observations of Holst and his coworkers[9] that a diet of oats alone or of oats and heated milk or oats and dried cabbage caused scurvy in the guinea pig, whereas oats and fresh milk or oats and fresh cabbage maintained the animals in health. Our own experience confirms theirs with respect to the fact that oats as the sole food will induce scurvy and that oats and fresh cabbage will adequately nourish the guinea pig. We have observed, as have recently Jackson and Moore,[10] that a large percentage of all guinea pigs kept on an oat and milk diet developed scurvy. Holst, we believe, overemphasized the protective powers of milk, either heated or unheated, as an antiscorbutic. He attributed the protective property of cabbage to the presence of a specific antiscorbutic substance which was destroyed by drying.

Since an exclusive oat diet induces the development of scurvy we were led to suspect that the oat kernel might prove to be a natural food deficient in at least one of the dietary essentials postulated by Funk; *viz.*, the antiscorbutic "vitamine." We therefore carried out a series of feeding experiments with rats, in which the oat kernel was fed in the following ways to a series of groups of rats:[11] (1) rolled oats alone; (2) rolled oats, purified casein; (3) rolled oats, fat-soluble A (in butter fat); (4) rolled oats, suitably constituted salt mixture to correct the inorganic

deficiencies of the oat; (5) rolled oats, protein, and fat-soluble A; (6) rolled oats, protein, and salts; (7) rolled oats, fat-soluble A, and salts; (8) rolled oats, casein, fat-soluble A, and salts. It was believed that the outcome of these feeding trials, which extended throughout the entire growing period beyond the time of weaning, would throw light on the question of the existence of a specific protective substance such as Holst, Funk, and others have assumed. Milk fat does not protect against scurvy[12] so we were not in any of the above described diets supplying any hypothetical food essentials known to Holst other than those contained in the oat.

We observed in the animals fed these diets no evidence of scurvy, but so far as we are aware this disease has not been observed in the rat. *Among all the combinations enumerated only Ration 8 was successful in inducing growth over a long period.* These results showed conclusively that *for the rat* the only deficiencies of the oat kernel from the dietary standpoint were found in the protein, inorganic, and fat-soluble A factors, and that as far as concerned unidentified substances of the water-soluble class, all which were necessary for complete growth and well-being in the rat were present in the oat kernel. If the explanation of Holst and Funk is correct that scurvy is the result of the *lack of a specific substance*, it becomes necessary to make the further assumption that the guinea pig and man require this protective substance, since both suffer from the disease, whereas certain other species, as the rat, do not require this complex as a dietary component. The only alternative is to conclude that scurvy is in reality not a deficiency disease in the sense in which this term has been employed during the past ten years.

Further to test the questions involved in determining the etiology of scurvy we fed to guinea pigs the following diets, both of which we have shown to be entirely adequate for the rat throughout the entire growing period.[10]

Lot 508.		Lot 273.	
Wheat embryo (extracted with ether)	33.0	Ground maize	50
Salt mixture 314	11.0	Alfalfa flour	30
Dextrin	46.0	Peas (heated)	20
Butter fat	5.0		
Casein	5.0		

Charts 1 and 2 illustrate the failure of the guinea pig to grow or long remain alive on these diets, in marked contrast to the complete nutrition of rats during nearly the whole of the period of growth beyond the suckling stage, and the maintenance of good health over a period covering 9 to 10 months. In fact one family of rats was restricted absolutely to Ration 273 and distilled water only, through four generations without impairment of vigor. Iodine was furnished once each week. These results, we believe, prove beyond a doubt that chemical sufficiency in a food mixture, as shown by feeding experiments with other species, will not necessarily mean that that food will adequately nourish the guinea pig. Nothing can possibly be lacking in the mixture of milk and oats, for some individuals take this diet, over a period of several months at least, without showing signs of the disease or of malnutrition, while others develop it within a few weeks (Chart 3).

Chart 3 illustrates the remarkable difference in the ability of individual guinea pigs to tolerate a rolled oats and fresh whole milk diet. Three of the four in this group lost weight rapidly after a brief initial gain and died after 5 to 7 weeks, with all the symptoms of scurvy. No. 2, however, is still in normal condition after 20 weeks on this diet and has increased in weight from 315 gm. to 575 gm. These records are typical of the behavior of a large number of guinea pigs which we have confined to an oat and milk diet.

It is much more rational to attribute the development of scurvy to some other cause than a deficiency of some protective "vitamine" in the diet. Chart 3 represents the typical results of keeping guinea pigs on an oat and milk diet. Most of them die early with scurvy; a few do not develop the disease. On autopsy of animals found dead with scurvy we usually found the stomach and small intestine empty or nearly so, indicating that they had not eaten for a day or two before death. The cecum was greatly distended with putrefying feces. There were usually no feces in the lower end of the large intestine. The cecum is extraordinarily large and delicate in this species, and the idea came to us that the development of scurvy in the guinea pig was due principally to retention of feces in the cecum.

The following line of reasoning will, we believe, account for

all the observed facts: The guinea pig can thrive only on a diet possessing such physical properties as will lead to the formation of bulky easily eliminable feces. Diets containing such succulent vegetable substances as green grass, cabbage, and carrots along with grains serve to keep them in good health. Diets such as oats alone cause injury not only because of poor constitution with respect to several dietary factors, but because oats lead to the formation of pasty feces which the animal is unable to remove from its delicate cecum. An impacted cecum, the seat of putrefaction, may cause injury to the cecal wall, sufficient to permit the invasion of the tissues by bacteria, or the animals may perhaps be injured primarily by the absorption of toxic products of bacterial origin which possess the peculiar pharmacological property of destroying the walls of the capillaries in those regions where hemorrhage is observed in scurvy. Infection of the hemorrhagic areas would then be secondary. Jackson and Moody[13] observed the presence of a diplococcus in the congested joints in guinea pigs suffering from the disease, and the assumptions made above may well account for the observed facts. They found that pure strains of these organisms inoculated into the circulation of guinea pigs and rabbits living under ordinary conditions (a mixed diet consisting of green vegetables, hay, and oats) gave rise in most instances to hemorrhagic and other lesions in the joints, muscles, lymph glands, or gums. The observation of Jackson and Moore[12] that a cream diet and a diet of olive oil added to milk produced a "fat constipation" with early death further supports the point which we wish to emphasize; *viz.*, that the undue retention of feces is the primary cause of the development of experimental scurvy in the guinea pig. Secondarily, toxic products of bacterial origin or the invasion of the body by bacteria are causal factors. If this theory can be supported by experimental facts it follows that in the guinea pig scurvy can no longer be looked upon as it has been in the past, as due to deficiency of a hypothetical antiscorbutic substance.

Obviously the conclusive demonstration of the validity of this hypothesis is peculiarly difficult. The protection of animals against scurvy, or their relief after the disease is established, by means of diets containing either natural foodstuffs or extracts of these, is always susceptible to interpretation on the assumption

that the proper "vitamine" was administered. The experimental data presented in this paper form, however, a conclusive line of evidence which proves that scurvy in the guinea pig is not a deficiency disease in the sense in which Holst, Funk, Hess,[14] and others have regarded it.

Since the diets described in Charts 1, 2, and 3 are all entirely adequate as far as can be shown by biological tests, but since owing to unfavorable physical properties guinea pigs are in general unable to tolerate them, we sought to find means which would tend (*a*) to depress the growth of microorganisms in the digestive tract and (*b*) to facilitate the elimination of feces, in the hope that by such aids the guinea pigs might be protected against scurvy, or relieved of the disease when continued on the milk and oat diet which, both in our experience and in that of Jackson and Moore, tends so definitely to permit the disease to develop. To this end we have restricted guinea pigs to a diet of rolled oats and fresh whole milk daily together with the following additions:

A.1. Dosage with 1 cc. of 0.1 N citric acid daily.

2. Inclusion of 0.2 per cent of sodium benzoate in the diet.

B.1. Inclusion of 8 mg. of phenolphthalein in a 50 gm. portion of the food consumed by each animal each week.

2. Dosage with liquid petrolatum at intervals varying from once a week to daily.

3. Administration of 1 cc. of orange juice to each animal daily, by means of a medicine dropper.

4. Administration daily of 1 cc. of orange juice neutralized with sodium hydroxide.

5. Administration daily of 1 cc. of orange juice which was neutralized as in No. 4 and heated 1 hour at 15 pounds' pressure.

6. Incorporation with the food of a salt mixture made up in imitation of the inorganic content of the edible portion of the orange. The average citric acid and sugar content of the orange was added.

The efficiency of orange juice as an antiscorbutic may well be accounted for by its content of sodium and potassium citrates, both of which possess laxative properties, together with the tendency of the citric acid to discourage the development of bacteria as long as it remained unabsorbed.

The results of these experiments are shown in Charts 4 to 11. It cannot be said that the citric acid served as a protective agent (Chart 4). It is of great importance, however, in connection

with the theory of the etiology of scurvy, that one of the guinea pigs in this group is still in excellent condition after 20 weeks, whereas others have died after 2 to 7 weeks on this diet. Those few animals which possess pronounced physical vigor tolerate longer the effects of a constipating diet. Like the history of one of the guinea pigs, No. 1 in Chart 3, this bolsters up the contention that an oat and milk diet causes malnutrition, not because of deficiency, but because of its constipating character.

Chart 5 shows the effect of incorporating sodium benzoate with a diet of oats and milk, the object being to discourage bacterial activity in the alimentary tract and thereby decrease the formation of toxic substances in the cecum. The addition of sodium benzoate did not, of course, remedy the constipating effects of the diet. The curves represent a group which was given 0.2 per cent of sodium benzoate. It cannot be insisted upon that the substance protected the animals, but it is highly probable that it did for two have remained healthy over a much longer period than is usual on the oat and milk diet. Since, however, some animals can withstand the diet for even longer periods than are covered by these experiments beneficial effects of sodium benzoate in the diet are not demonstrated.

Three of the seven animals given phenolphthalein have remained in excellent condition during 18 weeks and have steadily increased in weight. We feel confident that this laxative exerted a pronounced protective action on the animals. Their coats were glossy and they appeared to be exceptionally well nourished. This we have not seen in so pronounced a degree in guinea pigs fed on an oat and milk diet without modification. The three very small ones were too delicate to withstand the diet even when phenolphthalein was administered. The incidence of scurvy was delayed, however, even in these. The growth of one of these animals from 375 to 600 gm. during 14 weeks and its present excellent condition is alone sufficient to eliminate completely the possibility that an oat and milk diet is lacking in any chemical complex which could be considered an antiscorbutic substance.

Chart 7 offers definite and convincing evidence that scurvy is in reality the sequel to retention of feces in the cecum. The curves represent the records of six guinea pigs fed the oat and milk diet. Three were dead with scurvy by the end of the 4th

week. Two, Nos. 2 and 4, were unable to stand on the 31st day and showed badly swollen joints and hemorrhage of the gums. Their joints were very sensitive and the animals cried when touched. They were each given 1 cc. of liquid petrolatum daily. Although death regularly follows quickly when guinea pigs come to this condition these two animals gradually improved in appearance and ability to move their legs, and by the 10th day both were able to walk. Thereafter the amount of oil administered was reduced to 1 cc. each week. On the 18th day both were in good condition and showed no signs of lameness. The rate of dosage of oil remained at 1 cc. per week during the following 8 weeks. Both animals increased in weight, one from 260 to 465 gm., and it was at the end of the 23rd week apparently in as good condition as if its diet had included a succulent vegetable. After the end of the 8th week of oil treatment the dosage was increased to 2 cc. each week. After the beginning of the 24th week the animal was given the straight oat and milk diet without the oil. It lost weight rapidly and died 4 weeks later.

The other animal, No 2, suffered a fracture of the femur at the end of the 8th week after the oil treatment was begun, and was killed. It was in good condition and active when killed. Both animals were confined throughout the experiment strictly to the oat and milk diet which gave them the disease.

We have seen about sixty guinea pigs die within 3 to 6 weeks on a diet of rolled oats and milk, and the extensive records of Jackson and Moore add evidence which cannot be questioned that the mortality is very high on this diet. We have not yet seen a case where an animal has recovered or temporarily improved when continued on the oat and milk diet after the development of the disease when no remedial measures were employed. There is small room for doubt that the recovery of these two animals was the result of facilitating the elimination of feces through the lubrication of the tract with oil.

Charts 8, 9, and 10 show the results of feeding (*a*) orange juice fresh, (*b*) orange juice neutralized, and (*c*) orange juice neutralized with sodium hydroxide and heated in an autoclave at 15 pounds' pressure for 1 hour. In all cases the animals appear to have been benefited by the addition. It should be remembered, however, that a few individuals are capable of continuing to

grow slowly and escape scurvy on a diet of oats and milk with no addition of protective substances.

Human scurvy is apparently more readily relieved by orange juice than is the disease in the guinea pig. This may well be accounted for by the greater disability of the guinea pig in the possession of so large and delicate a cecum. We are inclined to attribute the beneficial effects of orange juice to its laxative action and perhaps also to a specific influence upon the growth of certain types of organisms, which, from the observations of Jackson and Moore, appear to be concerned in the production of the disease.

Another instance of nearly complete recovery may be mentioned. A guinea pig fed rolled oats and carrots, which had been heated in an autoclave at 15 pounds' pressure for 1 hour, developed scurvy after 21 days. When it was very thin and frail and showed marked stiffness of the hind legs, it was treated with 1 cc. of castor oil alternating daily with petroleum oil. After 2 weeks it had so far recovered as to hop about and showed the normal alertness. At this point 5 per cent of liquid petrolatum was incorporated in the oats. 2 weeks later it died. The food intake could not be accurately judged, but apparently it had eaten but little food because of its oil content.

We do not assert that liquid petrolatum is so efficient a protective agent as to correct entirely the injurious effects of an oat and milk diet.

There are in Charts 3 and 7 curves of growth practically as good in the case of individuals on a plain oat and milk diet and the same *after being cured of scurvy by petrolatum administration* as we have secured from an oat and milk diet supplemented with orange juice (1 cc. daily) (Chart 8). Even when orange juice was given daily two out of five failed to grow, and died early. We believe this result confirms the theory that the physical properties of this food supply are unfavorable for this species.

Chart 11 shows two records of particular interest in making clear the cause of scurvy. In Period I a group of four guinea pigs were fed rolled oats and heated carrots *ad libitum*. The carrots had been heated for 1 hour in an autoclave at 15 pounds' pressure. After this treatment the physical properties of the carrots are so modified that they no longer serve as effectively

to protect against scurvy as do fresh carrots. Nos. 3 and 4 had the swollen joints and inability to walk characteristic of scurvy at the end of 4 weeks. The remaining two did not show signs of the disease until they had been confined to the diet 12 weeks. No. 2 was in very bad condition, showing swollen joints, weakness, and labored breathing. At this point (Period 2) 34 gm. of a mixture of salts, citric acid, and cane sugar made up in imitation of the content of these substances in the edible portion of the orange, were combined with 966 gm. of rolled oats, and this mixture together with heated carrots was furnished in place of the plain rolled oats and heated carrots supplied during Period 1. Three of the animals recovered completely on this modified diet, but No. 3 always showed slight symptoms of scurvy. There can be no other interpretation placed on this result than that the laxative action of the salt mixture aided in producing a more hygienic condition of the digestive tract. The recovery of the remaining three is described in the legend to Chart 11.

With a plain oat and milk diet the results of feeding the "artificial orange juice" were not quite so satisfactory as with the oats and heated carrots. The latter doubtless serve in some degree to modify the intestinal flora, so that a remedial agent less effective serves to protect against scurvy. However, three out of eight guinea pigs which received this diet lived 8 weeks before showing signs of scurvy.

There is such great variation in the vitality of guinea pigs as shown by their reaction to experimental diets that uniform and consistent results cannot be expected in work of the character here described.

The observations reported in this paper furnish definite support for the idea that scurvy in the guinea pig is not the result of the deficiency of a specific protective substance. When considered in connection with our studies of the nutrition of the rat, in which we have made clear the chemical sufficiency of the diets employed, it becomes necessary to offer a new interpretation as to the etiology of experimental scurvy in the guinea pig. Our interpretation, that the first cause of the disease is associated with the retention of feces owing to diets of unfavorable physical character and debility of the digestive tract through stretching and contact with irritating and toxic putrefaction products of

bacterial origin, is we believe supported by adequate experimental data. This interpretation is entirely in harmony with the bacteriological studies of Jackson and Moody and of Jackson and Moore.

The significance of this interpretation is far reaching. It removes from the list one of the syndromes (scurvy) which has long been generally accepted as being due to dietary deficiency. It is of course not possible to say with confidence at the present time that human scurvy has the same origin as has the same symptom complex in the guinea pig, but the undoubted fact that some infants develop the disease while taking milk which forms a wholesome food for others supports the belief that the etiology of the disease is the same in both species.

In studies reported elsewhere we have shown that the entire seed in the case of the wheat,[4] oat,[11] maize,[6] rice,[1] pea,[15] beans,[16] millet seed,[17] and typical plant leaves such as alfalfa,[7] clover, and cabbage, contain in every case a liberal supply of all of the chemically unidentified dietary essentials which a ration must contain to make it complete, other than that which is supplied liberally by such fats as egg fat and butter fat (fat-soluble A). Certain of these plant products, as the seeds of flax and millet, and the leaves of plants as far as we have studied their nutritive properties in properly planned rations, likewise contain satisfactory amounts of the fat-soluble A as well. Since the fat-soluble A has been clearly shown not to be concerned with the development or relief of either polyneuritis in birds or of scurvy in either man or the guinea pig, this dietary factor and any functional or histological changes which may be caused by a deficiency of it in the diet, otherwise satisfactory, can be left out of further consideration here.

15 to 30 per cent of any of the seeds and leaves enumerated above serve to furnish an adequate amount of all dietary essentials of the water-soluble group which come under the classification of "vitamines," to meet the needs of the rat throughout the entire period of growth. This fact, together with convincing evidence that scurvy is not in reality a deficiency disease in the sense of being caused by a lack of specific protective substance, warrants an attitude of skepticism regarding the validity of the "vitamine" theory of the etiology of such other diseases as

pellagra, rickets, etc., which have been attributed to specific dietary deficiency.

When examined critically in the light of the extensive data collected in our laboratory, none of the experimental work conducted for the purpose of demonstrating a specific starvation for a still unidentified chemical complex in causing pellagra is at all convincing. Koch and Voegtlin[18] have compared the chemical changes in the nervous system of rats and monkeys fed such restricted diets as (*a*) corn germ cake, (*b*) equal parts of corn meal and sweet potato, (*c*) corn meal, and (*d*) raw carrots. They have pointed out the similarity of the changes observed with those seen in pellagrins. It should be clear to any one who is acquainted with the facts now available that their results are susceptible of an entirely different interpretation, and indeed must be otherwise interpreted as to the cause of the failure of function which precedes the anatomic changes which their chemical methods reveal. In the papers cited we have demonstrated the exact nature of the dietary deficiencies of a long list of natural foodstuffs in a wholesome condition (see especially reference 7) and have made it clear that malnutrition of the gravest character may follow the consumption of monotonous diets, restricted as to source and even including a wide variety if of strictly vegetable origin. In none of the cases of malnutrition which we have as yet observed is it necessary or even permissible in explaining their causes to postulate the existence of more than a single dietary essential (vitamine) of a water-soluble nature. The theory that the essential factors in a complete diet are (1) protein of suitable quality and quantity, (2) an abundance of available energy in the form of protein, carbohydrate, and fat, (3) a suitable inorganic content, (4) a sufficient content of the fat-soluble A, and (5) of the water-soluble B, proves adequate to account for the observed facts.

The extensive experimental data at present available support the belief that the water-soluble B contains but a single substance which is physiologically indispensable, rather than a series of such substances as is demanded by the "vitamine" hypothesis of Funk. Provisionally we have adopted this view, and predict that further inquiry will establish what is now all but demonstrated; *viz.*, *that unfavorable proportions among the well recognized*

constituents of the diet as well as of the two but recently appreciated ones, together with unsatisfactory physical factors and injury wrought through the agency of microorganisms inhabiting the alimentary tract, will account for all the observed types of pathological functioning which are referable to errors in the diet.

We believe there is satisfactory evidence in support of the view that polyneuritis is caused by a deficiency of a specific substance (our water-soluble B) in the diet. We have repeatedly observed the curative effects of antineuritic preparations from various sources, when administered to polyneuritic birds or rats, and accept the explanation of Funk as to the etiology of this disease. This is, we believe, the only known "deficiency disease" in the sense in which this term has been employed in recent years.

Since diets containing liberal amounts of butter fat (fat-soluble A) permit the development of scurvy, rickets, and polyneuritis there would seem to be but one syndrome, pellagra, which one might possibly refer to a shortage of this second unidentified dietary factor. There is, however, not the slightest evidence that there is any reason to attribute pellagra to this cause. Of the profound importance of proper amounts and relationships of the inorganic constituents of the diet our published results have furnished many examples. This, together with proteins of poor quality taken regularly at low planes, and an inadequate supply of fat-soluble A, has contributed to nutritive failure in all diets described by Goldberger and his associates as being employed by peoples where the incidence of pellagra is high. There is therefore no reason whatever why we should assume as Voegtlin,[18] Goldberger,[19] Funk,[8] and others have done that pellagra is due to a lack of a specific unidentified dietary factor, a "vitamine."

We shall report later a systematic study now being made of the nature of the dietary deficiencies of a number of fairly complex mixtures of natural foodstuffs.

The charts follow.

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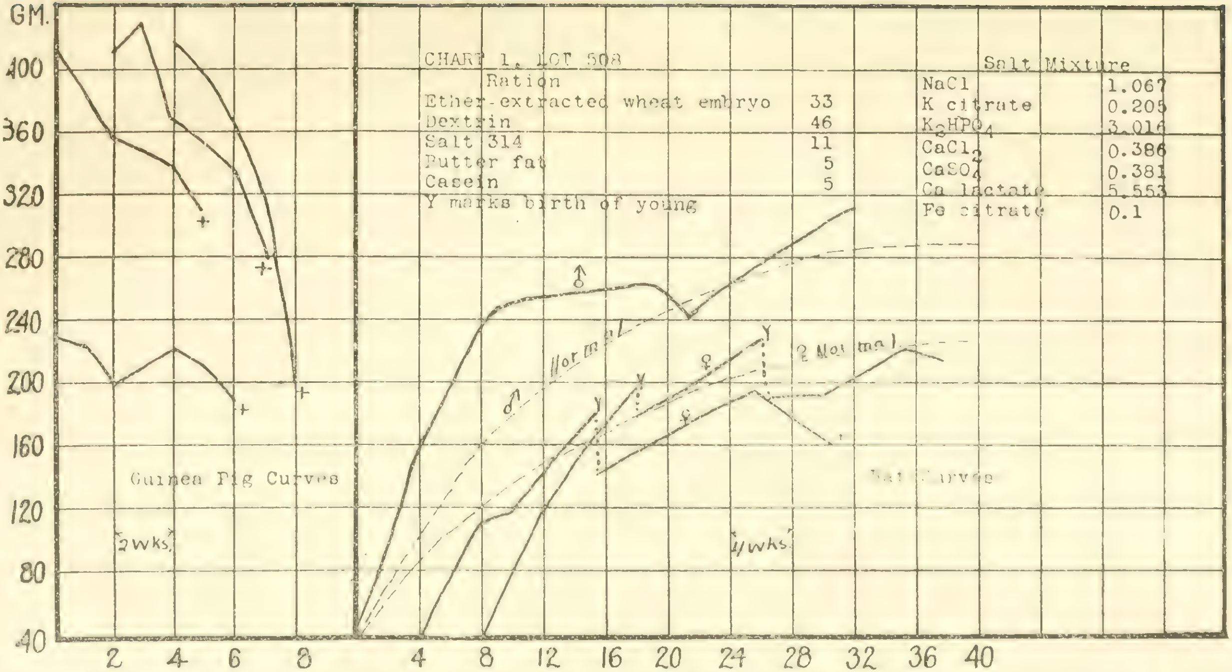

Chart 1. Illustrates the remarkable difference in the value of the same food mixture for the rat and the guinea pig. Rats thrive throughout the entire growth period and are able to produce young on this diet, whereas guinea pigs lose weight rapidly and die. We attribute this result to the necessity for having in the food of the guinea pig such substances as will form bulky easily eliminable feces. Succulent vegetables are especially effective in this respect.

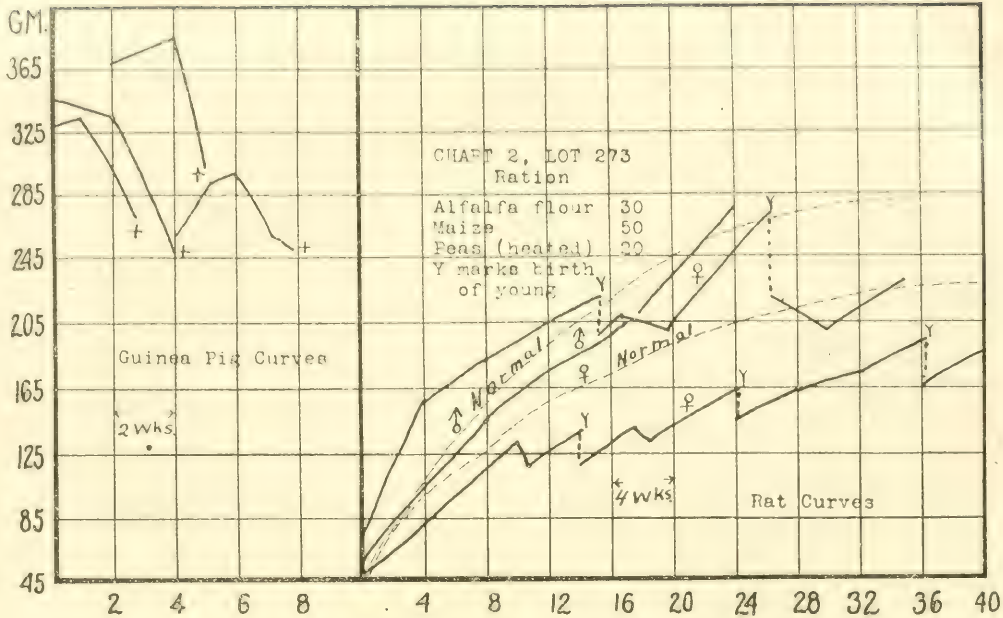

CHART 2. Shows another instance of a ration which suffices to maintain the rat in good health during four generations, whereas guinea pigs are unable to maintain health when restricted to the same food mixture. There is no deficiency in a chemical sense in this diet. The debilitating effects on the guinea pig are, we believe, the result of the physical character of the feces being such as render it difficult for the animal to empty its cecum. Scurvy does not develop on this diet or that described in Chart 1. This suggests that the character of the bacterial flora of the cecum is concerned in the production of this disease (compare Chart 3).

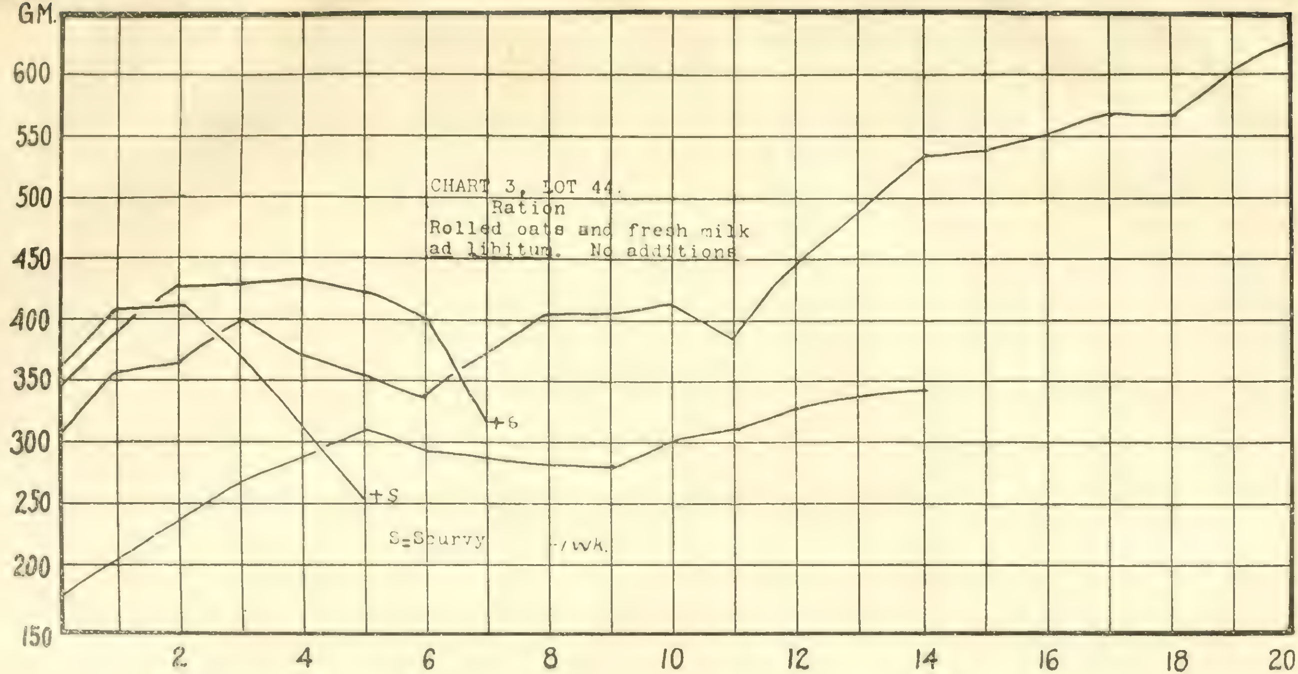

Chart 3. Illustrates the typical behavior of guinea pigs on a diet of rolled oats and fresh whole milk. Most of the animals develop the typical lesions of scurvy in 3 to 6 weeks. Young rats take a monotonous diet of rolled oats and milk and grow well. A few guinea pigs are able to maintain health and grow steadily, though not at the maximum rate on this diet, which is incontrovertible evidence that it is not deficient in any chemical complex. Scurvy in these animals must be accounted for in some other way than as being the result of a lack of a specific antiscorbutic substance in the diet. Both milk and oats are constipating foods and lead to impacted cecum in the guinea pig. We attribute to this cause (*a*) injury to the cecal wall with possible invasion of the tissues by bacteria, and (*b*) the absorption of toxic products of bacterial origin which may be possibly associated with the causation of capillary hemorrhage.

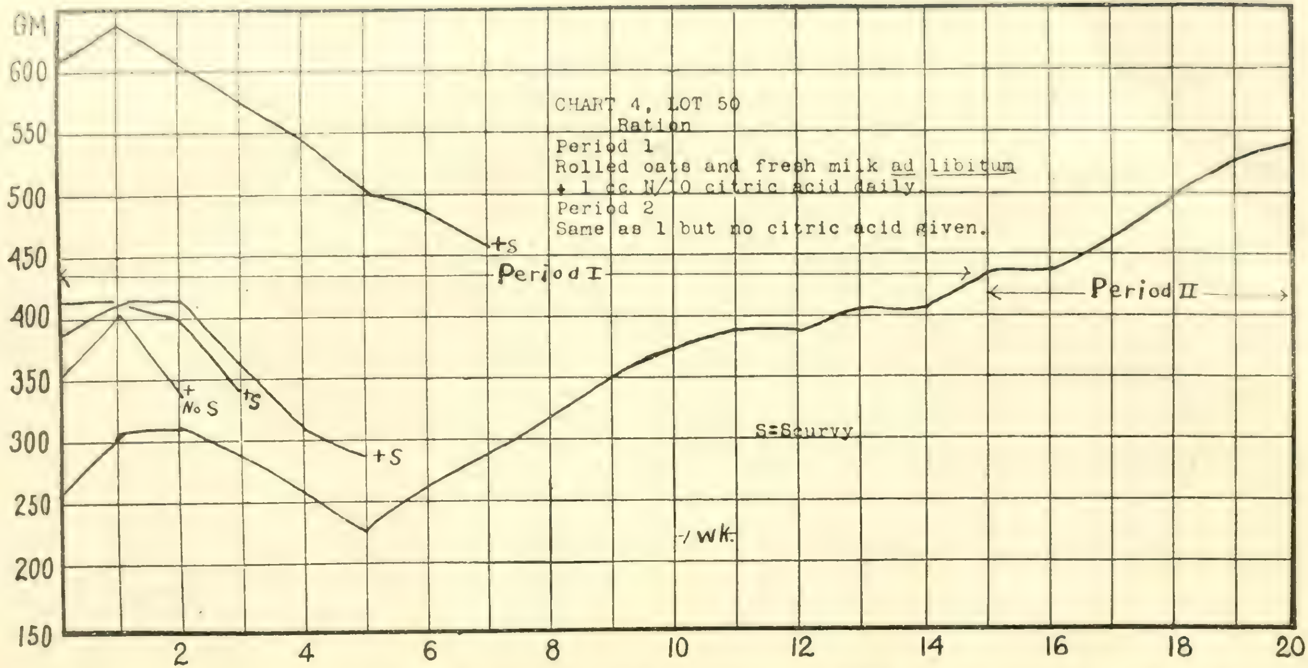

CHART 4. We attempted to discourage the growth of microorganisms in the intestine by the administration of citric acid daily. It cannot be said to have protected the animals to a noticeable degree. The curves are shown here to illustrate how on a rolled oat and fresh milk diet one guinea pig in a group of five maintained good health and more than doubled its weight during a period of 20 weeks, which emphasizes our contention that this diet is not deficient in "vitamines," an explanation which Funk and others have put forward to explain the etiology of scurvy. It is a more plausible explanation to attribute the disease to an unhygienic condition of the intestinal tract, especially of the cecum, as the result of restriction to a constipating diet rich in protein and fat, which favors the development of an injurious bacterial flora.

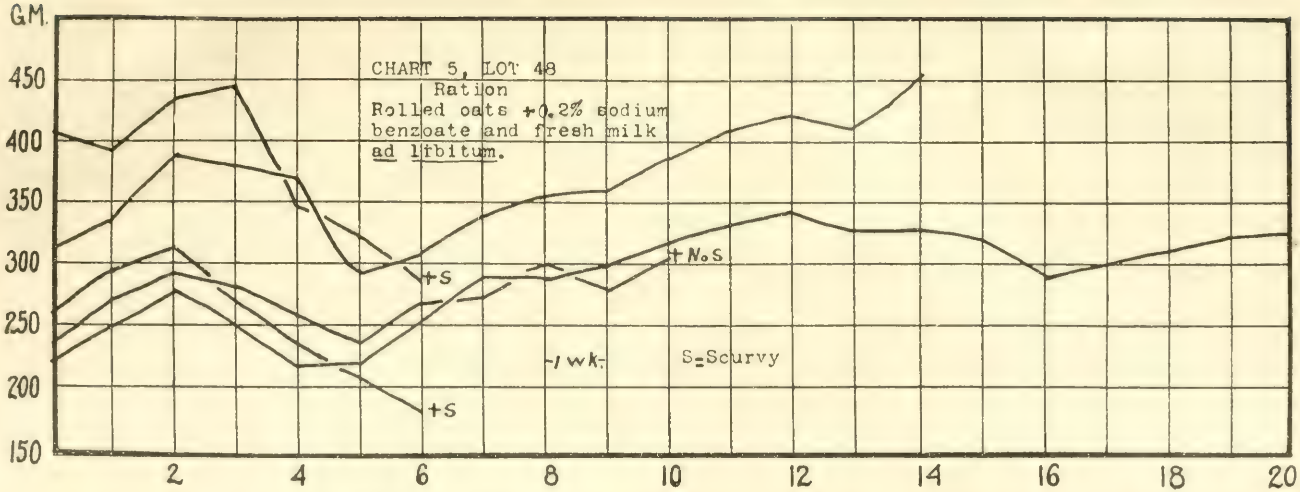

CHART 5. This illustrates again an attempt to depress the growth of bacteria by feeding a diet of rolled oats and fresh milk together with 0.2 per cent of sodium benzoate. This addition appears to have exercised a slight protective effect, for three of the five animals lived much longer than is usual on this diet without the sodium benzoate. In this group, as in those of Charts 3 and 4, are individuals which maintained a fair degree of health and increased in body weight over a long period. This makes it evident that those which develop scurvy did so from some cause other than lack of an antiscorbutic substance in the oat and milk food. Sodium benzoate, of course, did not tend to improve the physical properties of the food.

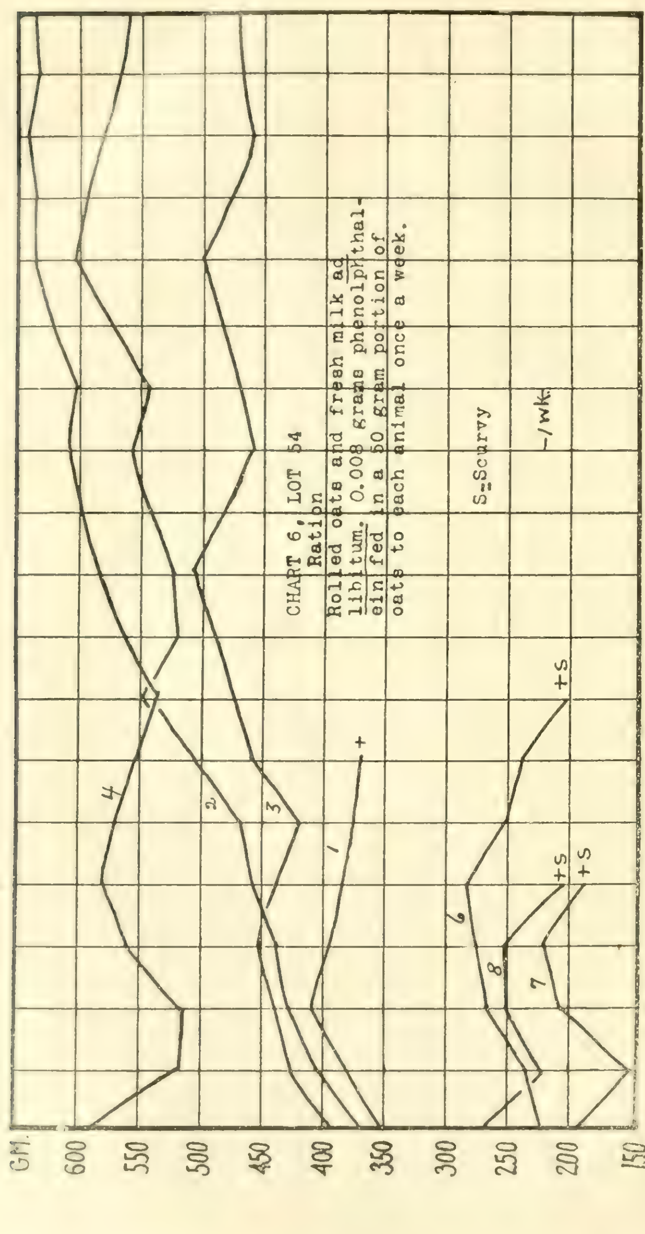

CHART 6. This shows the results of feeding guinea pigs a diet of oats and milk and of giving them regularly a suitably adjusted dose of phenolphthalein to counteract by its cathartic action the tendency to retain feces in the cecum. These animals were apparently benefited to a considerable degree by this treatment for they not only grew well but after 18 weeks they are exceptionally sleek and fat. Two, the smallest of the seven, died after 4 weeks and showed slight signs of scurvy.

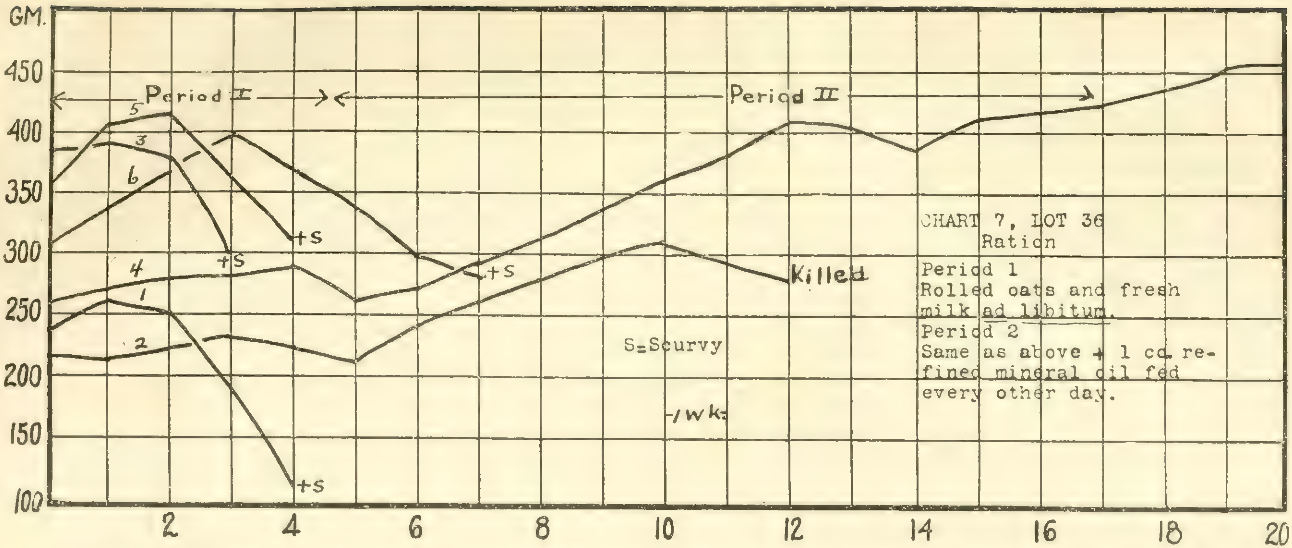

Chart 7. In Period 1 the animals in this group were fed rolled oats and fresh whole milk *ad libitum*. One died of scurvy at the end of 3 weeks and two others at the end of the 4th week. Nos. 2 and 4 were unable to walk on the 31st day and showed the swollen joints and hemorrhagic gums characteristic of scurvy. They were given liquid petrolatum daily, and gradually recovered completely and became as spry and sleek as if on a diet of green vegetables, while continued strictly on the diet of oats and milk which gave them the disease. These cases represent actual cures of animals near death with scurvy, by treatment with a substance, liquid petrolatum, which cannot possibly carry an antiscorbutic substance, and argues strongly against the idea that scurvy is a "deficiency disease" in the sense in which that term has been employed within recent years.

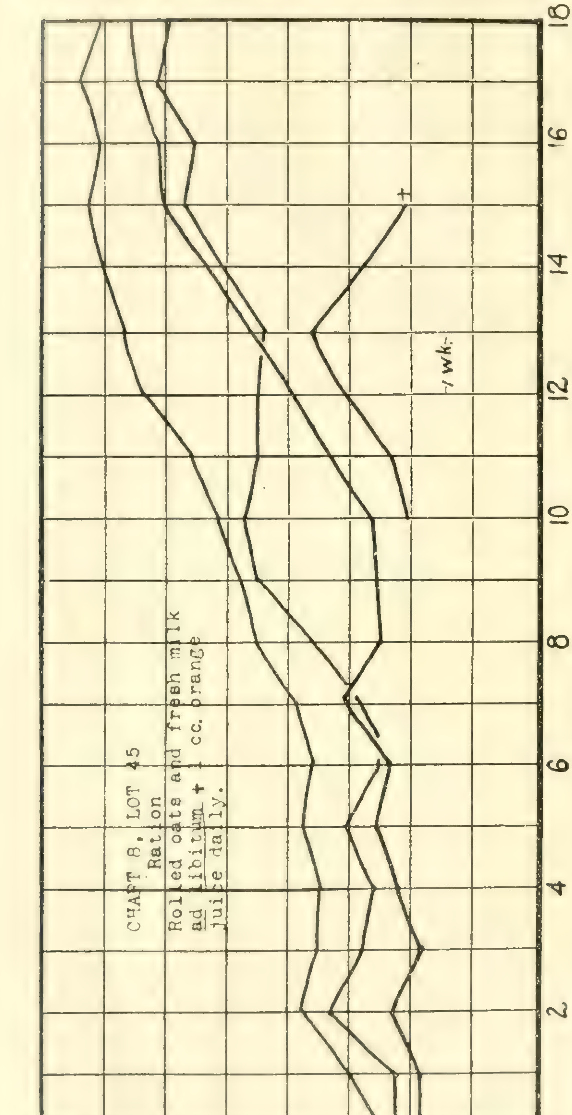

CHART 8. This shows the curves of four guinea pigs fed rolled oats and milk and given 1 cc. of fresh orange juice daily. Although this protects a high percentage from scurvy they are not able to grow normally. The oat and milk diet is chemically adequate, but its physical properties are not satisfactory for a digestive tract with the peculiar anatomical features seen in the guinea pig. The evidence presented in the charts in this paper indicates that the efficiency of orange juice as an antiscorbutic is not due to its containing a specific antiscorbutic substance, but rather to its effect on the physical character of the feces and on the character of the bacterial flora of the cecum.

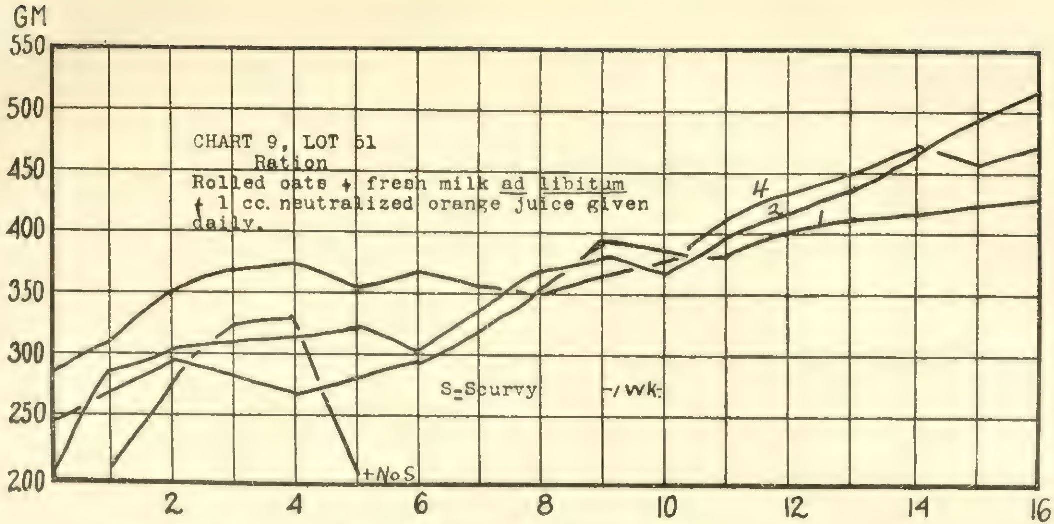

Chart 9. This shows the growth curves of four guinea pigs on a rolled oat and milk diet with 1 cc. each of orange juice daily. The latter was neutralized with sodium hydroxide. This protects the animals against scurvy. Suitable doses of substances such as phenolphthalein (Chart 6) or liquid petrolatum (Chart 7) likewise serve to protect a considerable number, and a few animals are able to take oats and milk without addition of protective substances and grow fairly well without developing scurvy.

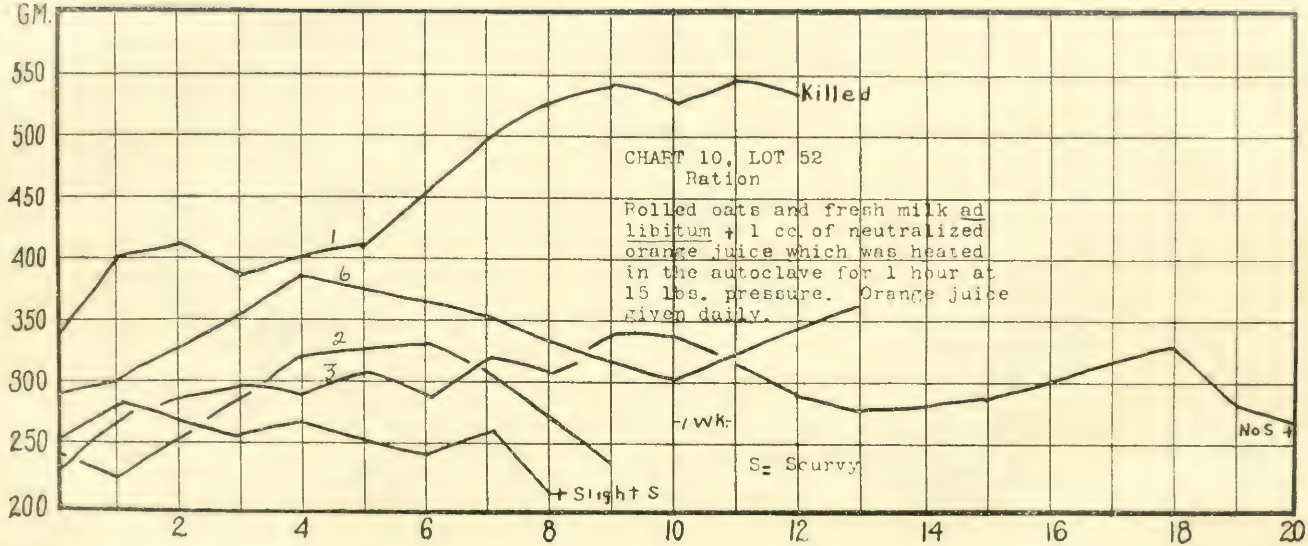

Chart 10. These guinea pigs received an oat and milk diet together with 1 cc. of neutralized orange juice which had been heated in an autoclave at 15 pounds' pressure for 1 hour. The variation on the behavior of animals on an unmodified oat and milk diet makes it impossible to decide whether the orange juice had lost anything in protective power through heating. The other data afforded by our experiments lead us to the conclusion that orange juice protects because of its aiding elimination and modifying the intestinal flora rather than by furnishing a hypothetical antiscorbutic substance.

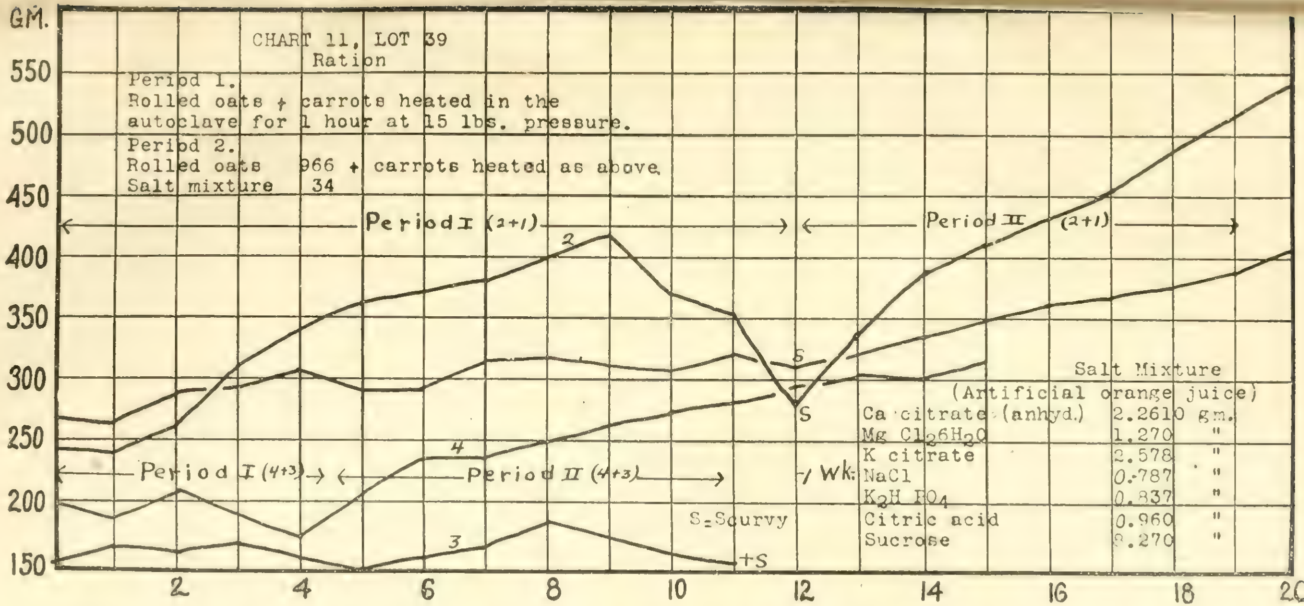

CHART 11. In Period 1 the animals of this group were fed rolled oats and carrots. The latter were heated for 1 hour at 15 pounds' pressure in an autoclave. Two of them (Nos. 3 and 4) had scurvy at the end of the 4th week, while Nos. 1 and 2 did not develop the disease until the 12th week. In Period 2, therefore, all had scurvy. In the second period the rolled oats were mixed wet with a salt, citric acid, and sugar mixture made up in imitation of the content of these in the edible portion of the orange.[20] 10 gm. of oats carried an amount of the mixture equivalent to 2 cc. of orange juice.

Guinea Pig 2 was in a very bad condition with scurvy, and breathed with difficulty. After the introduction of the salt, citric acid, and sugar mixture the animal improved rapidly and was at the end of 20 weeks in an excellent condition.

Guinea Pig 4 had lost the use of the hind legs and dragged them about. Its recovery was slower than that of No. 2, but it was in excellent condition 8 weeks after the modification of the diet.

Guinea Pig 3 was also in a critical condition at the end of Period 1. Although it did not gain in weight its condition greatly improved in Period 2. There still remained some stiffness in the hind legs, but it became alert and had a good appetite. It died 7 weeks after the beginning of Period 2. Its joints were somewhat swollen at the time of death.

THE INFLUENCE OF BILE ON PHENOL PRODUCTION.

By HARRY DUBIN.

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(Received for publication, June 5, 1917.)

We have found in our previous studies of phenol production that an increase in the formation of phenols, whether normal or pathological, resulted, with one exception, in an increased conjugation.[1] Here there was an increased phenol formation with an unchanged or even lessened conjugation, indicating that the bile plays some part in assisting the liver in its conjugating function. It was in the hope of throwing further light on this subject that the present investigation was undertaken.

Our methods of procedure and analysis have already been described.[1] After a period of normal observation, the dog was operated on[2] and a bile duct-ureter anastomosis was produced after the method of Pearce and Eisenbrey.[3] Bile was continually present in the urine, no evidence of jaundice being present at any time. In one instance the bile was removed from the urine by allowing it to stand with fullers' earth for 2 hours. However, on analysis this clarified urine gave the same figures as were obtained with the original urine. At autopsy the anastomoses were found to be intact. In Dog 3, an external bile fistula was produced whereby we were able to collect the bile so that the urine was free from it. The external fistula was produced as follows.

Small holes were cut for a distance of about 5 inches, and near one end, in the walls of a rubber tube about 15 inches long. Around these holes were tied a soft rubber bag, the rubber tube at this end being fitted with a small silver cannula. The silver cannula was inserted into the common bile duct and tied in place.

[1] Dubin, H., *J. Biol. Chem.*, 1916, xxvi, 69.

[2] For the operative work, we are indebted to Dr. J. E. Sweet of the Department of Surgical Research.

[3] Pearce, R. M., and Eisenbrey, A. B., *Am. J. Physiol.*, 1913, xxxii, 417.

The other end of the tube was led out through a stab wound and clamped off, so that the bile collecting in the soft rubber bag, which remains inside the body, could be drained off. The animal was bandaged to keep the rubber tube in place. As early as 24 hours after the operation an increased phenol production with an unchanged conjugation was obtained.

Table I presents a short résumé of work previously published, showing a comparison of results obtained under various pathological conditions, normal control periods being present in each case. The table shows the contrast in regard to the degree of conjugation in the bile dog as compared with the others.

TABLE I.

Phenol Elimination under Normal and Pathological Conditions.

Dog.	Phenols.			
	Free.	Total.	Free.	Conjugated.
	mg.	*mg.*	*per cent*	*per cent*
1.				
Normal	167	217	76	24
Bile duct cut	263	329	80	20
2.				
Normal	175	227	77	23
Intestinal obstruction	263	477	55	45
3.				
Normal	184	223	83	17
Pancreatic ducts cut	203	293	69	31
4.				
Normal	165	192	86	14
Eck fistula	205	213	96	4

Table II summarizes the results obtained in the present work. In all cases, although there is a very large increase in the phenol production, still the extent of conjugation remains practically unchanged. The case where bile was removed from the urine before analysis serves to rule out any influence due to the presence of bile in the urine. Furthermore, the external fistula dog tends also to show that the presence of bile in the urine plays no part in the large amounts of phenol found. Feeding of fresh liver or boiled liver with the idea of supplying possibly the lacking biliary

constituents had no effect on the quantity of phenols formed. It appears to us that the results obtained are primarily due to some definite rôle which the bile plays in the production of phenols and their conjugation.

TABLE II.

Influence of Bile on Phenol Production.

Dog.	Date.	Phenols.				Weight.	Remarks.
		Free.	Total.	Free.	Conjugated.		
	1916	*mg.*	*mg.*	*per cent*	*per cent*	*kg.*	
1	Nov. 1						Bile duct-ureter anastomosis.
	" 26	287	345	83.3	16.7	16.25	Some urine lost.
	" 27	234	291	80.4	19.6	16.19	
	" 28	336	420	80.0	20.0	16.13	
	" 29	414	489	84.6	15.4	16.38	
	Dec. 4	385	482	80.0	20.0	17.07	
	" 5	396	489	81.0	19.0	17.07	
	" 12	414	512	80.8	19.2	16.90	Boiled liver fed.
	" 13	414	512	80.8	19.2	16.93	" " "
	" 14	427	528	80.9	19.1	17.00	" " "
	" 14	414	518	80.0	20.0	17.00	Portion of this day's urine treated with fullers' earth for 2 hours to remove bile.
	" 15	538	673	80.0	20.0	17.05	Fresh liver fed.
	" 16	598	752	79.6	21.4	17.14	" " "
	" 17	538	673	80.0	20.0	17.13	" " "
	1917						
2	Jan. 30	185	229	80.8	19.2	11.82	
	" 31	192	229	83.8	16.2	11.95	
	Feb. 6						Bile duct-ureter anastomosis.
	" 28	269	332	81.2	18.8	10.20	
	Mar. 1	287	367	81.1	18.9	10.12	
	" 2	298	369	80.7	19.3	9.92	
	1917						
3	Feb. 28	179	220	81.3	18.7	14.22	
	Mar. 1	185	225	82.2	17.8	14.08	
	" 2	182	223	81.8	18.2	14.42	
	" 8						External bile fistula.
	" 9	267	330	81.0	19.0	13.8	

Two things are to be considered: first, the observation that in the absence of bile from the intestine, large amounts of phenol are produced; and second, that in spite of the unusually large amount of phenols found, the degree of conjugation remains normal. With the second point must be considered the possibility that the liver in the absence of bile in the intestine does not function properly, or that the phenol is formed so gradually that the liver is not called upon to do extra work. In our opinion, both of these factors mentioned are instrumental in the unchanged conjugation.

As to the first consideration, namely, that in the absence of bile from the intestine large amounts of phenol are produced, various explanations suggest themselves.

First, there is the theory that the bile exerts some antiseptic action. Under normal conditions, bacteria play an important part in bringing about disintegration of nitrogenous materials of the intestine. Even in health there is considerable variation in the character and amount of decomposition thus brought about. Metchnikoff and others believed that the products of decomposition developing within the bowel had much to do with organic changes and ill health. For a considerable time it has been claimed that the bile had a direct antiseptic effect upon the growth of the intestinal bacteria and by this means undue putrefaction was held in check. Newer studies, however, have indicated that, with few exceptions, bile exerts little influence on the growth of bacteria. Still, it has been established that under conditions of bile stasis, when little or no bile flows into the intestine, abnormal decomposition of the intestinal contents occurs. Roger,[4] investigating the problems incidental to the determination of the influence of bile upon intestinal bacteria and their secretions, found that bile inhibited the growth of anaerobes but had no marked influence upon the growth of other intestinal forms. He found, however, that the bile had an inhibitory effect upon the fermentative secretions of various bacteria as well as an influence in diminishing the toxicity of their products. These tests were carried out with various bacteria *in vitro*, while the antitoxic qualities of bile were observed *in vivo*, using rabbits. Roger concludes that the antagonism of bile to putrefaction is not so

[4] Roger, H., *Ann. Inst. Pasteur*, 1915, xxix, 545.

much dependent upon the inhibition of bacterial growth as upon its neutralizing effect on the fermentative secretions of bacteria. It is also possible that it not only destroys the bacterial ferments, but also renders inert their products of decomposition.

The second theory is what might be called the digestion activity of the bile. Bile salts have a solvent action on proteins, which property therefore acts as an aid to speed up digestion, with the consequent diminished decomposition. Kingsbury[5] shows that the presence of bile makes possible a much greater soap formation from the fatty acids liberated during digestion than could otherwise be the case with an alkali as weak as sodium bicarbonate—in opposition to the erroneous assumption of Pflüger that the alkali of the small intestine available for the neutralization of fatty acids is sodium carbonate instead of sodium bicarbonate.

SUMMARY AND CONCLUSIONS.

It is established that in the absence of bile from the intestine large amounts of phenol are produced, this increase being unaccompanied by an increase in the conjugation.

The unchanged conjugation may be due either to impaired liver function, or a gradual slow production of phenol, or to a combination of both.

The increased phenol production in the absence of bile from the intestine is due probably to the increased decomposition of the intestinal contents brought about by the lack of digestion activity, and diminished inhibition of bacterial fermentative secretions.

In an earlier report,[1] it was suggested that the results of the analysis of the urine for both free and conjugated phenols might be a valuable index of intestinal putrefaction. The results reported here point to a method of differentiating the factor of biliary obstruction.

[5] Kingsbury, F. B., *J. Biol. Chem.*, 1917, xxix, 367.

THE URIC ACID CONTENT OF THE BLOOD OF NEW-BORNS.*

By F. B. KINGSBURY and J. P. SEDGWICK.

(*From the Departments of Physiology and Pediatrics of the University of Minnesota, Minneapolis.*)

(Received for publication, May 9, 1917.)

It has long been known that the uric acid excretion in the urine of children during the first few days of life is both relatively and absolutely high. Reusing (1) determined the daily excretion of this substance during the first 7 days of life of six normal children and found that it reached its maximum of 0.083 gm. on the 3rd day. He used the analytical method of Hopkins (2). Schloss and Crawford (3), using the procedure of Folin and Shaffer (4), determined the uric acid excretion of nine infants each day up to and including the 9th day, and found in four cases that the excretion reached its maximum on the 3rd day, in two cases on the 2nd day, in two cases on the 1st day, and in one case on the 4th day. Their results are confirmatory of Reusing's. Schloss and Crawford also pointed out that the urinary excretion of phosphorus was highest during the first 3 days of life and believed that this indicated a common source of origin of phosphoric acid and uric acid, the nucleoproteins. They did not, however, determine the fecal phosphoric acid excretion. The purine content of the colostrum they found to be too slight to account for the high uric acid excretion.

It is well known that during this period when the uric acid excretion is highest rapid morphologic changes occur in the blood of the new-borns, the disappearance of the nuclei of many of the red cells, the change in proportion from a predominating number of polynuclear neutrophilic cells to a corresponding predominance of lymphocytes (Carstanjen (5)), and a striking decrease in the leukocyte count of the peripheral blood confirmed by Schloss and Crawford and others. Schloss and Crawford refer to the work of Goldscheider and Jacob (6), Schulz (7), and Bohland (8) as evidence that the fall in the leukocyte count in the peripheral blood may not be due to an actual destruction of these cells but to a redistribution, and therefore regard this question as so far unsettled. It is not

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* A preliminary report of this work was made at the annual meeting of the Federation of American Societies for Experimental Biology, December, 1916, in New York.

the purpose of the present paper to discuss this point which will be taken up more in detail in a later publication.

That the increased excretion of uric acid follows the increased metabolism of nucleoprotein is so well known that it is unnecessary to refer to the large literature on this subject. This fact makes attractive the view that the increased excretion of uric acid in the urine of new-borns is due, in part at least, to a destruction of leukocytes at this time, but as noted above, the fact of such a destruction is at present disputed. The cause of this increase in the uric acid output must, in the light of our present knowledge, be referred in part at least to an increased metabolism of nucleoprotein material, but where in the body this is taking place must be regarded as an unsettled question.

The occurrence of uric acid infarcts in the form of neutral, acid, or a mixture of both urates in the kidney of the new-born during the first few days of life is an established fact, and it was shown by Schloss and Crawford that uric acid infarct elements were present in all the urines of the new-borns referred to.

The purpose of this investigation is to determine whether or not the high uric acid excretion during the first few days of life is accompanied by a simultaneous increase of this substance in the blood. While it seemed probable that this would be found to be the case, there was, nevertheless, a reasonable doubt concerning this question which could be settled only by actual determination of the uric acid content of the blood.

Methods.

Benedict's (9) modification of the original Folin-Denis (10) method for the determination of uric acid was tried, but found more tedious than the ater modification of Myers, Fine, and Lough (11). The latter modification with certain minor changes was used throughout the work. It was found that alumina cream could be made much more simply and with the attainment of a more suitable product by the method of Tracy and Welker (12) than by the directions given by Myers, Fine, and Lough. This substance was washed free from sulfate ions by decantation and concentrated by means of the centrifuge before using. 4 cc. of this were used in all cases except in a few where larger amounts were necessary. It was found that there was always a certain amount of insoluble material present in the concentrated blood filtrate after the removal of proteins when this had been transferred with the washings to the centrifuge tube. Since the presence of this residue interfered with the colorimetric readings at a later stage in the procedure it was customary to remove it. After developing the blue color in the centrifuge tube, and before diluting it to volume, the tube was centrifuged, the supernatant liquid poured off into

the glass-stoppered graduated cylinder (a similar one was used for the standard colored solution), the precipitate washed once with about 5 cc. of water, recentrifuged, and transferred to the cylinder with the main bulk of blue solution. This operation usually required not more than $1\frac{1}{2}$ minutes (the standard solution remaining undiluted the same length of time). By this means it was always possible to obtain an unknown blue solution that was perfectly clear and therefore easy to match with the standard. The standard color was that made by 0.4 mg. of uric acid and was usually diluted to 30 cc., a dilution that gave the most satisfactory depth of color when the colorimeter was set at 20 mm. The unknown color was diluted to the volume that gave approximately the same shade as the diluted standard, the regular procedure of Myers, Fine, and Lough, and much more satisfactory in our hands than the older procedure in which the dilution was more or less arbitrarily fixed, with the result that one had frequently to compare colors that were of much greater degrees of difference, and therefore harder to match in the colorimeter.

It was noticed by Curtman and Freed (13) that the standard uric acid solution of Benedict and Hitchcock (14) slowly deteriorated, particularly if the laboratory in which the solution was kept cooled off at any time. This is confirmed by our experience, and it was customary to check the standard solution from time to time against a new solution. It was found that the standard would hold its strength for at least 10 days in our laboratory, but that while some solutions kept for a month or more, it was unsafe to assume that any standard more than 10 days old was still serviceable.

Daylight was used whenever possible in reading the colorimeter, but in a few cases this was impossible without letting the analysis stand over at some stage until the next day. While there is no evidence at our disposal to indicate that a delay in the analytical procedure vitiates the results, it nevertheless happened that in most of the few cases in which no color, or very little, was obtained with the uric acid reagent, the analysis had been delayed. As to the cause of these failures, which have been previously noted by Morris (15), we have no definite information. In order to avoid this possible source of error it was customary to use an artificial source of light in a few analyses. This consisted in a 100 watt nitrogen burner of "Daylite" glass enclosed in a box having a ground glass window. It was found that pure uric acid solutions gave the same relative colorimetric readings with this light as with daylight, and that it was fully as easy to compare the colors accurately. The blue solutions produced by the blood uric acid, however, gave readings about 0.2 mm. higher than when compared with the same standard (at 20 mm.) under exactly the same conditions by daylight. For this reason the artificial light was used only when absolutely necessary to avoid letting the analysis stand over until the next day. Not more than ten colorimetric readings in the whole number of analyses recorded in this paper were made with this light.

Collection of Blood Samples.

Blood was drawn through a sterile hypodermic needle from the superior longitudinal sinus of the new-born into a glass syringe and expelled with force into a small weighing bottle containing 0.1 gm. of potassium oxalate. The most convenient method of introducing the oxalate into the bottle was found to be to pipette 1 cc. of a 10 per cent solution of this substance and dry it with gentle heat on an electric hot plate. This gave a film of salt easily penetrable by the blood. In some few cases where clots were formed the liquid portion of the blood was transferred to the casserole, the clot then ground thoroughly to a homogeneous paste with pure sea sand, and quantitatively transferred to the casserole with the main bulk of blood.

The weights of blood samples used in the analyses recorded in this paper varied from about 9 gm. to 18, but the usual sample, particularly of infant blood, was about 13 to 14 gm.

Placental blood was collected as soon as possible after cutting the cord, which was done late, *i.e.*, after the cord had stopped pulsating. This blood and that of the mother were also collected in weighing bottles containing 0.1 gm. of potassium oxalate. The maternal blood was drawn from a vein in the arm of the mother at the time of parturition. All the new-borns which served as subjects in this investigation were normal.

Uric Acid Content of Maternal and Placental Blood.

A separate series of analyses were made to determine the uric acid in maternal and placental blood in order to obtain a basis of comparison for the uric acid content of new-born blood. Several of the analyses recorded in this series had been made when it was learned that Dr. J. M. Slemons of New Haven, Connecticut, was working on the placental blood as a separate problem. In a private communication he clearly established his priority in this matter (the relation of the uric acid content of maternal blood with that of placental blood) and reported that he had found identical values for this substance in maternal and placental blood. His figures have not yet been published, nor are they known to us.

Our figures for this series of analyses are shown in Table I. It will be noted that the average values for sixteen determinations of the uric acid content of maternal and placental blood are identical, confirming the independent observation of Slemons.

Table II shows the results obtained with new-borns. In some cases the infants were given water (marked + in the table)

in addition to their regular diet of breast milk. It will be noted that the content of uric acid in the blood the first 3 or 4 days after birth is higher than that of the blood of the same newborn at birth. In seven cases there is a marked decrease in this value at the end of 8 to 11 days from the value obtained (in each case with the same infant) 3 to 8 days earlier, and also from the value at birth.

TABLE I.

Case.	Uric acid in 100 gm. of blood.	
	Maternal blood.	Placental blood.
	mg.	*mg.*
B.		2.9
Dr.	3.5	2.8
P.	3.6	3.3
Mc.	2.2	2.4
N.	2.9	2.7
M.	3.1	2.8
R.	3.1	3.5
K.	2.7	
T.	3.3	2.8
Ha.	5.0	5.0
Wa.	3.1	3.2
Hi.	2.8	2.6
Da.	2.9	3.1
Hu.	2.1	2.5
We.	3.3	
V.		3.1
A.	2.1	2.3
S.	3.7	4.1
Average..............	3.1	3.1

Table III shows the average values for blood uric acid each day of life from birth to the 5th day and during the period of the 8th to the 11th days. It will be noted that during the period between the 2nd and 3rd days (48 to 71 hours) the value reaches a maximum. A much larger series of analyses would be necessary to fix beyond question the day on which this maximum average value is reached, if, on account of individual variations, this could be established at all. During the first 3 or 4 days of life

TABLE II.

Case.	Sex.	Uric acid in 100 gm. of blood.																Weight of new-born at birth.	Water given new-born.
		Placental blood.	Blood of new-born.																
			0–23 hrs.		24–47 hrs.		48–71 hrs.		72–95 hrs.		96–119 hrs.		120–134 hrs.		8–11 days.				
		mg.	*hrs.*	*mg.*	*hrs.*	*mg.*	*hrs.*	*mg.*	*hrs.*	*mg.*	*hrs.*	*mg.*	*hrs.*	*mg.*	*days*	*mg.*		*gm.*	
Mu.	♂		9	4.2														3,445	—
Hf.	♀				26	3.7												3,340	+
Ka.	♀		23	2.9														4,140	—
Mr.	♂		8	4.9											10	1.3		3,445	+
Ki.	♂	1.7	9	2.7														3,120	+
R.	♀	3.1									96	3.7						3,600	—
Ke.	♂	1.9			46	4.0									9	3.5		3,375	—
Bo.*	♂	3.6			46	3.8												2,280	+
Sc.	♂	2.8			44	3.0												2,900	+
Be. †	♂												120	2.1				3,375	—
Be. †	♀												120	3.4				3,230	—
Mc.	♂				39	5.1												4,060	—
Ba.	♂	4.3							72	4.1								3,280	+
Ml.	♂	3.1							72	4.2								3,930	+
Sp.	♂		23	2.9														3,160	+
E.	♀	2.1			36	3.6									8	1.7		2,740	+
T.	♀	2.5									96	2.6			8	1.4		3,160	+
Mo.	♂	2.7											120	2.5	8	1.1		3,190	+
L.	♂								72	3.8								3,220	—
Dh.	♀								72	2.8								3,415	+
Hl.	♂						48	4.3										3,650	+
Sa.	♀						48	4.7										3,340	—
A.	♂						48	2.5										3,750	+
Z.	♀				38	3.7									11	1.1		3,420	+
P.	♂				36	3.0												3,910	—
Ha.	♀						48	3.7							10	1.4		2,780	+
Bu.	♂														10	1.5		2,870	—
Sm.	♀								72	2.7			134	3.4				3,330	—
Mt.	♀						65	3.4										3,020	+
Dv.	♂								72	2.9								4,220	—
P.$_1$	♂								76	3.7								3,845	—

* Premature.

† Twins.

the uric acid content of the new-born blood is higher than that of the placental and maternal blood.

From a maximum of 3.9 mg. the blood uric acid falls off slowly to 2.9 mg. on the 5th day and then very rapidly to 1.6 mg. by the 8th to the 11th days. This value agrees with the 1.3 to 1.7 mg. per 100 cc. of blood found by Liefmann (16) for thriving children from 9 weeks to 14 months of age, on a pure milk diet.

TABLE III.

Blood of new-born.	Uric acid in 100 gm. of blood.	No. of analyses on which average is made.
	mg.	
At birth*	3.0	42
0— 23 hrs. old	3.5	5
23— 47 " "	3.6	8
48— 71 " "	3.9	5
72— 95 " "	3.5	7
96—119 " "	3.2	2
120—134 " "	2.9	4
8— 11 " "	1.6	8

*This is the average value for the maternal and placental figures.

CONCLUSION.

Our finding that there is a parallelism between the high uric acid content of the blood of new-borns and the high excretion of this substance during the first 3 or 4 days of life is indirect evidence supporting the findings of Wells and Corper (17) and others (in opposition to the results of Schittenhelm and Schmid (18)) that human fetal tissues possess no uricolytic power, for it would be difficult to imagine so great a production of uric acid if the tissues themselves possessed the power to destroy it. Whether the decomposition of nuclein material, which must be looked upon as the cause of this uric acid increase in the blood, is related to the striking changes in the partition of the white corpuscles taking place at this time, or to nuclein destruction in other parts of the body, or to both, must be left to the future to decide.

We wish to thank Dr. N. O. Pearce and Dr. A. G. Alley of the Pediatrics Department for obtaining for us the blood samples of new-borns, and also the members of the obstetrical staff of the Elliot Memorial Hospital for obtaining the samples of maternal and placental blood.

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THE HYDROGEN ION CONCENTRATION OF THE ILEUM CONTENT.

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(Received for publication, May 30, 1917.)

It has been shown by the senior author that the reaction of the duodenal contents of infants is distinctly acid (pH 3.1). A few determinations of the duodenal contents of adults, that had been exposed to air for hours, showed them to have become slightly more alkaline than the blood (pH 7.7) but whether they are alkaline in the body was not determined. One sample of adult duodenal contents that was strongly colored with bile was about as acid as the stomach. According to Foà, dogs' bile and calves' bile is acid (pH 5.4 and 6.5), and it seems probable that the neutralization of the acid from the stomach is accomplished by the pancreatic juice alone. Pancreatic juice is usually mixed with bile in the common bile duct, but the presence of bile in the duodenum may *not always* indicate that pancreatic juice is present also. We opened the abdominal cavity of a dog several hours after a hearty meal and before the stomach was nearly empty. The ileum was empty except for some residue packed in its lower end. The duodenum contained bile-stained fluid of an acid reaction. Auerbach and Pick found the contents of the small intestine of dogs to be slightly alkaline, but it is not clear that some CO_2 was not lost from the samples. Although the exact pH of the small intestines of men and dogs may be uncertain, it is probably higher (less acid) than the duodenal contents of infants. Since we did not have the opportunity to study the contents of the ileum of infants, we used pups, and found the contents of the ileum to be slightly acid throughout the nursing period and later on a diet of solid food. The contents of the ileum were less acid than those of the infants' duodenum (except possibly during the first week), as shown in the following table of seven pups of the same litter.

Age, *days*	4	9	11	16	18	42	46
pH of ileum	5.7	6.75	6.34	6.3	6.1	6.15	6.0

The last two pups took only solid food and the acidity of the stomach was as high as in the dog. The younger pups nursed at very frequent intervals; the pH of the stomach contents varied from about 5.5 to 6 and the pH of the ileum was nearly the same as that of the stomach at the same time. The low acidity of the stomach is due to the acid-binding power of the milk.

These experiments offer a suggestion as to the conditions in the infant. The gastric juice of the infant is nearly as acid as that of the adult. The period of 4 hours between feedings allowed the stomach to become empty and to accumulate a very acid fluid which ran down into the duodenum. We suppose such a process would take place in the pup with 4 hour feedings. We have no evidence, however, to indicate whether the reaction of the infant's ileum is the same as or different from the duodenum.

The determinations were made with the hydrogen electrode described by McClendon and Magoon, the tip being inserted through a small hole cut in the ileum and the electrode filled by pressing on the gut. The temperature of the room was electrically regulated to 20°. The Leeds and Northrup potentiometer was the same as in preceding experiments, but a Leeds and Northrup (Type R) galvanometer of about 4,000 ohms was used in place of the capillary electrometer. The oscillations of this galvanometer cause much waste of time, but we damped the oscillations at intervals in the following manner. A key was used that opened the circuit when pressed down half way and closed it when pressed all the way down, but short circuited the galvanometer when released. When the galvanometer is short circuited, the potential energy of the twisted suspension or the kinetic energy of the moving coil is transformed into electrical energy and hence into heat in the highly resistant circuit, and hence the rate of movement of the coil is brought almost to zero.

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LIGHT PRODUCTION AT LOW TEMPERATURES BY CATALYSIS WITH METAL AND METALLIC OXIDE HYDROSOLS.

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(Received for publication, June 1, 1917.)

INTRODUCTION.

Reactions which are capable of producing light at low temperatures are of considerable interest because of the probable similarity to the processes occurring in the luminous organisms. Lophin, esculin, several essential oils, and certain alcohols and aldehydes have been known to luminesce in the presence of sodium and potassium hydroxide, all of them requiring, however, a higher concentration of alkali than is compatible with life (Radziszewski, 1887, 1880; Dubois, 1901; Trautz, 1905).

More recently, lophin and esculin have been found by Ville and Derrien (1913) and by Dubois (1913) to luminesce with blood and hydrogen peroxide, the hemoglobin acting as an oxidase. Harvey (1916) has described a reaction which shows the general character of these luminescent reactions. He found that the "oxidation of a mixture of pyrogallol and hydrogen peroxide by vegetable oxidases occurs with the production of light." In this reaction the mixture of hydrogen peroxide and pyrogallol corresponds to the oxidizable material occurring in the luminous organism, the vegetable oxidase to the oxidizing enzyme, both of which must be present in order that oxidation may proceed with production of light. The oxidases used by Harvey were usually potato or turnip juice or a 1 per cent extract of ox blood. He found that perceptible light could be produced with concentrations of pyrogallol as low as 0.000031 M, becoming brighter with increased concentration up to 0.00012 M, above which there was little change. The light increased in brightness with increased concentration of oxidase and also with a rise in temperature, a faint light being produced at 0°, a bright light at 10°C. The oxidase was destroyed in the course of the reaction and was therefore not a real catalyst but one factor in what Bayliss (1915) calls a "coupled reaction." Other oxidases were tried and found to give light, among which were horseradish and sweet potato juice and the extracts of several invertebrates. Certain inorganic substances, such as

potassium ferrocyanide, ferric chloride, and potassium permanganate, could take the place of the vegetable oxidase.

The suggestion was made by Bach (1897) and later by Kastle and Loevenhart (1901) that the action of oxidases depends on the formation by these bodies of unstable peroxide-like substances which can give up their oxygen in turn to an oxidizable substance present in the cell. Bach and Chodat (1903) separated an oxidase into two constituents, one of a peroxide nature which they called "oxygenase," another which could excite the oxygenase to activity, called a "peroxidase." While the origin and exact nature of the oxygenase are still somewhat doubtful, it is generally accepted that biological oxidations are brought about by oxidases which consist of two substances, oxygenases and peroxidases.

In many reactions the behavior of plant peroxidases has been found to be closely imitated by platinum and palladium sols. The effect of these metals, in the colloidal state, on the decomposition of hydrogen peroxide (Paal, 1907), the union of hydrogen and oxygen (Paal, 1916, *a*), and the oxidation of carbon monoxide to carbon dioxide (Paal, 1916, *b*) has been studied, as well as the transfer of oxygen from hydrogen peroxide to gum guaiac by Reed (1916, *a*) who observed that different samples of colloidal platinum and silver behaved differently toward guaiac, in some cases bringing about oxidation directly, in others only in the presence of hydrogen peroxide.

These facts suggested the possibility that certain of the metal and metallic oxide sols might be able to take the place of the vegetable oxidases in the so called luminescent reactions. Experiments have therefore been made to determine the effect of various colloids on the oxidation of pyrogallol, the results of which are presented in this preliminary paper.

EXPERIMENTAL.

The reaction used in this study of luminescence was the one described by Harvey (1916) in which a half and half mixture of 0.01 M pyrogallol and 3 per cent hydrogen peroxide (McKesson and Robbins, commercial, neutralized by the addition of 0.1 N sodium hydroxide) was added to an equal volume of the vegetable oxidase solution. 1 cc. each of the pyrogallol and hydrogen peroxide were placed in a test-tube together and a series of such tubes arranged in a rack directly in front of the observer. Directly in front of these tubes a corresponding series was placed containing 2 cc. of the colloidal sol whose peroxidase activity was to be tested. All observations were made after a period of

TABLE I.

Catalysis of Light Production by Colloidal Metals and Oxides.

Sol.	Method of preparation.	Character of light produced.*
Silver.	Suspension of freshly precipitated silver oxide in water and reduced by a stream of hydrogen gas at 70°C.	–**
"	" in 0.1 per cent dextrin.	–
"	" " 0.1 " " potassium stearate.	–
"	" in 0.1 per cent dextrin + 0.001 N NaOH.	+
"	Paal's, protected, sodium protalbate (Paal, 1904).	++
"	Bredig arc dispersion, 4 amperes, in conductivity water.	++
"	" " in 0.001 N NaOH.	+++
Platinum.	" " dispersion in conductivity water.	+++
"	" " " in 0.001 N NaOH.	+++
Palladium.	" " " "	+++
Gold.	" " " "	+
Copper, copper oxide.	" " " "	–
Ferric oxide.	Peptonization.	–
Manganous oxide.	From freshly precipitated hydroxide by hydrogen at 70°C.	+
" "	Paal's, protected, sodium protalbate.	–
Silver "	" " " "	–
Copper "	" " " "	–
Nickel "	" " " "	–
Chromium "	" " " "	+
Cobalt "	" " " "	+++

* The intensity of the light produced has been described as faint, fair, or bright; faint light has been indicated by +, fair light by ++, and bright light by +++. If no light was visible it has been indicated as –.

** Negative results in the case of the silver sols prepared by reduction of the freshly precipitated oxide were probably due in part to the fact that only very dilute sols could be obtained in this way. The addition of dextrin, potassium stearate, sodium glycocholate, etc., resulted in the formation of a more concentrated sol, presumably because of the lowering of surface tension.

15 minutes in total darkness. In these reactions the maximum light was usually developed within ½ minute after the time of mixing and was quite transient, having disappeared within 2 or 3 minutes.

Observations were first made on a variety of sols, prepared by different methods, in order to determine which metals and oxides were effective in the catalysis of this reaction. The results are shown in Table I.

In order to compare the activity of platinum, palladium, silver, and gold in the transfer of oxygen, sols of these metals were prepared in 0.001 N NaOH by the Bredig arc dispersion method. After letting stand for a day and decanting the solution away from the large particles which had settled out, the gross concentration was determined by evaporating a known volume of the sol and weighing the residue. By successively diluting these solutions with distilled water, the minimum concentration was found which would just produce visible light.

The effect of potassium stearate in increasing the intensity of the light was unexpected. Colloidal sols of gold, silver,

TABLE II.

*Minimum Concentration of Colloidal Metal Necessary to Produce Visible Light.**

Silver.		Platinum.		Palladium.		Gold.	
Concentration.	Intensity of light.	Concentration.	Intensity of light.	Concentration.	Intensity of light.	Concentration.	Intensity of light.
gm./cc.		*gm./cc.*		*gm./cc.*		*gm./cc.*	
0.000200	++	0.000120	+++	0.0000960	+++	0.00024	+
0.000100	+	0.000060	+++	0.0000480	+++	0.00012	+
0.000050	+	0.000030	+++	0.0000240	++	0.00006	−
0.000025	−	0.000015	++	0.0000120	+		
0.000012	−	0.000008	+	0.0000060	+		
		0.000004	+	0.0000030	−		
		0.000002	−				

* The values obtained here would doubtless depend to a considerable extent upon the size of the particles. The figure obtained for the minimum concentration of gold required to produce visible light would, therefore, be farther from that of platinum than appears from the above table since the particles in the red gold sol used were probably in the range of 20 to 30 $\mu\mu$ in diameter, while those of the platinum were larger, 40 to 50 $\mu\mu$.

platinum, and palladium were taken of the concentration which would just produce visible light and the effect was compared with that produced by the same concentration of metal in 0.5 per cent potassium stearate. In each case the light was markedly increased. Potassium stearate alone gave no light. Isocapillary solutions (stalagmometer method) of potassium stearate, sodium stearate, and potassium oleate, containing 0.000017 gm. per cc. of palladium were tried and it was found that potassium stearate increased the light greatly, sodium stearate increased it slightly, while potassium oleate cut off all light. The same relative effect was observed at 80°C., above the melting point of the stearic acid formed by hydrolysis.

TABLE III.

Effect of Protective Colloids in Inhibiting the Production of Light.

Dispersion medium.	Light produced.	
	Platinum sol, Bredig 0.000008 gm./cc.	Silver sol, Bredig 0.0002 gm./cc.
Water	+	++
Gelatin 0.5 per cent	−	−
Egg albumin 0.5 " "	−	−
Potassium oleate 0.5 " "	−	−
Saponin 0.5 " "	−	−
Potassium stearate 0.5 " "	+++	+++
Tannic acid 0.5 " "	−	−
Sodium glycocholate 0.5 " "	−	−
Gum arabic 0.5 " "	−	−
Agar 0.5 " "	−	+
Dextrin 0.5 " "	−	+
Gum tragacanth 0.5 " "	−	+

The addition of alcohols increased the light in general, the higher alcohols producing the greater effect in equimolar concentrations. Of those tried, capryl > amyl > butyl > propyl > ethyl > methyl. Ethyl and methyl gave results differing little from that in pure water. This action of the higher alcohols cannot be explained on the basis of lowering of surface tension alone, since even when isocapillary solutions were used (Lillie, 1916) the order of effect was amyl > propyl > methyl. Further investigation will be necessary before many of the observed phenomena can be explained.

DISCUSSION.

It has been observed by Harvey that the intensity of the light produced does not run parallel to the rate of evolution of oxygen. This has been confirmed by my experiments, since in some cases a violent bubbling off of oxygen occurred when little or no luminescence was present. It is evident, too, that the effect of the colloidal metal was not due entirely to the large surface exposed and the consequent adsorption of oxygen, since widely different results were obtained with metal sols of approximately the same degrees of dispersion. The colloidal metals which were most active in transferring oxygen from the hydrogen peroxide to the pyrogallol were those which are able to form unstable compounds with oxygen. Some interesting experiments along this line were described by Reed (1916, *a*), showing that when finely divided platinum (platinum black, incorrectly called "colloidal platinum") on an electrode is charged with oxygen by making it the anode, it can bring about oxidations directly. Platinum charged with hydrogen cannot oxidize directly, yet both are able to decompose hydrogen peroxide. The author (Reed, 1916, *b*) draws the conclusion that "although substances which act as oxidases or peroxidases usually decompose hydrogen peroxide, yet in the case of colloidal platinum they are quite independent."

It must be pointed out, however, that in the decomposition of hydrogen peroxide by colloidal platinum it is only the rate of a reaction already occurring which is changed, and that a sample of platinum black cannot be placed in contact with a solution of hydrogen peroxide without immediately taking up oxygen. I have observed, for example, that if finely divided platinum which showed no direct oxidase action, *i.e.*, was unable to blue gum guaiac, was placed in hydrogen peroxide for a few minutes and then removed and washed with four changes of distilled water, it did show direct oxidizing action by blueing guaiac solution. This was not due to an adsorption of hydrogen peroxide as such, as has been suggested by some authors, since the same result was obtained with colloidal platinum sols by bubbling air through them or, more slowly, by letting them stand in contact with air. Platinum, palladium, and silver sols, as prepared by the arc dispersion method in water, showed this presence of oxygen by blue-

ing guaiac directly without the presence of hydrogen peroxide. If a stream of hydrogen was bubbled through the sol for a few hours, the colloidal metal lost the power to oxidize guaiac directly but produced oxidation by hydrogen peroxide, or, if let stand in air for only a few minutes, the colloid took up oxygen and regained its oxygenase activity.

Bose (1900), after summarizing the work done previous to that time, concluded from the experimental evidence that the combination of hydrogen and oxygen with platinum, palladium, and gold was not due to the formation of compounds, but to solid solution, together with a certain amount of adsorption. He noted that, while the metal had a greatly differing capacity for hydrogen and oxygen, the total of absorbed and adsorbed gas became greater with subdivision of the metal, and that the difference between the capacity for hydrogen and oxygen became less. Later investigations have supported this view regarding the condition of the hydrogen, but have indicated the formation of a series of oxides of platinum and palladium. Lorenz (1906) noticed that the E.M.F. of cells with platinum electrodes differed widely among themselves and suggested that the E.M.F. of the oxygen electrode was determined by the formation of an oxide. He found that the oxides of cadmium, copper, silver, and iron gave an E.M.F. which was identical with that of the metal when measured under the same conditions. Wöhler (1905) studied the oxides of palladium and their behavior toward reducing agents, and found that palladium dioxide, although a strongly exothermal compound, was a better oxidizing agent, toward many substances, than molecular oxygen. He suggested that this behavior was due to the easy splitting off of atomic oxygen. Hydrogen peroxide reduces palladium dioxide much more readily than platinum dioxide, but the reverse order is true of the monoxides, platinum monoxide being easily reduced by organic acids or by hydrogen peroxide. Palladium metal catalyzed the decomposition of hydrogen peroxide more vigorously than palladium dioxide, which, in turn, decomposed the peroxide more rapidly than the monoxide. The authors concluded that the decomposition of the hydrogen peroxide was brought about, not by palladium monoxide or dioxide necessarily, but perhaps by an intermediate oxide.

It appears from these experiments that, in the case of the col-

loidal metals, catalase activity and the ability to transfer oxygen, *i.e.*, oxidase activity, are not at all independent but that both are dependent upon the ability of the metal to combine with oxygen to form an unstable complex which is a better oxidizing agent than either hydrogen peroxide or molecular oxygen. This combination is probably due to adsorption of oxygen on the great surface exposed, together with the formation of oxides as the result of this adsorption, the oxide being soluble in the metal. The oxidation of the pyrogallol in this reaction mixture was brought about, therefore, by both the adsorbed and combined oxygen, the proportion of adsorbed oxygen becoming greater with decreased size of particles. The colloidal metal is continually supplied with oxygen by the hydrogen peroxide, whose normal decomposition according to the equation

$$H_2O_2 \rightleftarrows \overset{+}{H} + \overset{-}{HO_2} \rightleftarrows \overset{-}{HO} + O$$

is accelerated by the removal of oxygen atoms. The metal-oxygen complex furnishes "active" oxygen by dissociating in contact with the pyrogallol.

SUMMARY.

White light was obtained by the oxidation of pyrogallol with hydrogen peroxide in the presence of certain colloidal metal and metallic oxide sols, closely resembling that produced by luminous organisms.

The action of the colloidal metal was similar to that of the vegetable oxidase, except that in the former case the catalyzer was not destroyed.

Visible light was produced by concentrations of colloidal platinum as low as one part in 250,000.

A platinum sol containing 0.0002 gm. per cc. produced a fair light at $-5°$C. and a bright light at $0°$C.

This catalysis was not due entirely to the high degree of dispersion of the metal or oxide and the consequent large specific surface, but was also, in part, dependent upon the ability of the metal to form unstable compounds with oxygen.

The production of light was inhibited, in general, by the presence of protective colloids such as gelatin and egg albumin.

Potassium stearate markedly increased the intensity of the light produced, the concentrations of the other substances remaining constant. A similar effect was observed in the presence of the higher alcohols such as capryl and amyl, suggesting the influence of surface tension.

The author wishes to express his appreciation of the assistance given by Drs. E. N. Harvey and Alan W. C. Menzies in this investigation. A closely related paper by Harvey, who has noticed independently that oxidation with light production may be brought about by colloidal silver and platinum, appears on page 311.

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BLOOD FAT IN DOMESTIC FOWLS IN RELATION TO EGG PRODUCTION.*

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(Received for publication, June 2, 1917.)

The natural color of the body fat of most hens and the fat in egg yolk is yellow. This color is due to a pigment of the xanthophyll group with a very little "carotin-like pigment," as shown by Willstätter and Escher in their isolation of it in crystalline form from eggs,[1] and identical with plant xanthophyll as shown by Palmer.[2] It was the disappearance of this color from the external parts of the hen's body that led the writers to make the present investigation. There is a correlation between egg laying activity and yellow pigment in the domestic fowl. Blakeslee and Warner[3] have shown that where the yellow in the ear lobes did not exceed 20 per cent in a given group of hens the per cent of birds laying at that time was high; but with birds having a higher amount of yellow there was a decline in per cent laying. The conclusion drawn by Blakeslee and Warner[4] was that "the laying removed the yellow pigment from the body for the production of eggs more rapidly than it could be replaced by the normal metabolism." With this fact in mind the writers believed that if the yellow pigment which was present in the hen's body previous to its laying was transferred to the egg yolk, in like manner the body fat which contains the yellow pigment would be taken from the body by the blood to assist in the build-

* By fat in this paper we mean the total ether extract composed chiefly of fats and cholesterol.

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[2] Palmer, L. S., *J. Biol. Chem.*, 1915, xxiii, 261.

[3] Blakeslee, A. F., and Warner, D. E., *Am. Naturalist*, 1915, xlix, 360.

[4] Blakeslee and Warner, *Science*, 1915, xli, 432.

ing up of the egg yolk. If this is the case a hen which is laying heavily should have blood much richer in fat than a hen that is not laying.

The object of this paper is to show the relationship of blood fat in fowls to (1) egg production, (2) presence of food in the alimentary tract, (3) color of legs, etc., and (4) sex.

Warner[5] has shown that the average amount of fat found in the high producing hens and hens that were laying was 1.426 per cent; in the low producers it was 0.886 per cent. This, however, was preliminary work and involved only ten hens.

These results were considered worthy of a further study. In October, 1916, eighty-two hens and twelve cockerels belonging to the Storrs Agricultural Experiment Station were used for the following work. All of the hens used for this work were White Leghorns that had just completed their 1st or 3rd year of laying, and their trap nest records were at hand. Among this group of females were found birds having high, mediocre, and low fecundity.

EXPERIMENTAL.

In considering various procedures for the determination of fat in blood the methods of Kumagawa and Suto,[6] Rosenthal and Trowbridge,[7] and Bloor[8] seemed to require too much time and manipulation without compensating advantages. A modified Soxhlet method for fat, similar to the one used by Gettler and Baker,[9] was used for this work.

About 5 gm. of blood were drawn from the basilar vein into a small test-tube. This blood was poured into a thin-walled lead dish of known weight, containing a mat of asbestos fiber, and the whole was quickly weighed. The dish and contents were then dried over phosphorus pentoxide *in vacuo* for 12 hours at 50–60°C., and kept over phosphorus pentoxide until extraction was

[5] Paper read before the Ohio meeting of the American Association of Instructors and Investigators in Poultry Husbandry, August, 1916; *J. Am. Assn. Instructors and Investigators in Poultry Husbandry*, 1916, iii, No. 1, p. 4.

[6] Kumagawa, M., and Suto, K., *Biochem. Z.*, 1908, viii, 212.

[7] Rosenthal, H., and Trowbridge, P. F., *J. Biol. Chem.*, 1915, xx, 711.

[8] Bloor, W. R., *J. Biol. Chem.*, 1914, xvii, 377.

[9] Gettler, A. O., and Baker, W., *J. Biol. Chem.*, 1916, xxv, 218.

made. After an 18 hour extraction the ether was evaporated from the extracted fat and the extraction flask dried over phosphorus pentoxide *in vacuo* to constant weight at 50–60°C. By the use of this process the extracted material was found to be completely free from blood pigments and only possessed the yellow color of fat when it was present in the larger amounts; otherwise the extract was colorless. The extraction as described above gave fat, cholesterol, and fatty acids, and probably also traces of other lipoids.[9] For the discussion in this paper the entire extracted material is called fat. The cholesterol was determined in the blood of a few laying and non-laying hens and also a few roosters, as shown in Table X. The average amount of cholesterol for these laying and non-laying hens was more uniform than the fat for these same groups, and no particular correlation could be traced. It is planned, however, to make a further study of the cholesterol and fat in the domestic fowl.

The method used for the determination of cholesterol was that used by Gettler and Baker, as directed by Autenrieth and Funk[10] and also by Bloor.[11] As outlined by the above method, the fatty extract was taken up with 5 cc. of chloroform to which were added 2 cc. of acetic anhydride and 1 cc. of sulfuric acid, and made up to 10 cc. with chloroform. The solution was then kept in the dark for 15 minutes and compared in a Duboscq colorimeter with a similarly treated standard solution of cholesterol in chloroform.

Table I shows that the total per cent of fat found in the blood of seventy hens was 28.467 per cent or a mean of 0.407. The per cent of fat found in the blood of hens is variable and the average per cent of fat found in this group of Leghorn females is much lower than that reported by Ingles.[12] The per cent of fat varies from 0.083 to 1.953, which is quite striking. The variation is due largely to the fact that the birds that had a high per cent of fat in their blood were all laying at the time the samples of blood were taken, whereas most of the others were not laying.

[10] Autenrieth, W., and Funk, A., *Münch. med. Woch.*, 1913, lx, 1243.

[11] Bloor, *J. Biol. Chem.*, 1916, xxiv, 227.

[12] Ingles, H., Manual of Agricultural Chemistry, London, 1908, 259. The amount of non-protein reported by Ingles would, however, include fat, mineral matter, and possibly some organic substances.

TABLE I.

Fat in the Blood of Seventy Hens That Had About Completed Their 1st Year of Laying.

No.	Fat.	No.	Fat.	No.	Fat.
	per cent		*per cent*		*per cent*
15B– 9	0.083	15B–52	0.180	15B–32	0.258
15	0.085	46	0.181	5L–67	0.272
13	0.088	5L–71	0.183	5L–62	0.300
54	0.114	68	0.187	15B–97	0.307
16	0.123	15B–55	0.187	5L–60	0.312
5L–54	0.123	58	0.188	15B–11	0.406
15B–56	0.124	20	0.189	5L–51	0.437
18	0.125	5L–64	0.191	15B–23	0.448
3	0.131	77	0.195	64	0.448
60	0.131	15B–80	0.198	5L–63	0.526
74	0.133	31	0.202	15B–24	0.541
49	0.138	36	0.202	39	0.625
5L–53	0.144	37	0.204	5L–70	0.735
15B–51	0.154	66	0.204	57	1.255
19	0.161	34	0.205	15B– 5	1.315
5L–66	0.166	25	0.223	89	1.319
15B– 1	0.167	62	0.228	5L–65	1.388
83	0.167	98	0.228	15B–90	1.641
5L–52	0.167	104	0.230	5L–59	1.779
15B–96	0.169	82	0.231	15B–57	1.864
5L–55	0.175	77	0.241	43	1.915
15B–38	0.178	91	0.242	12	1.953
99	0.179	5L–69	0.246	Total.	28.467
105	0.180	15B–28	0.253	Mean.	0.407

Table II shows the per cent of fat found in the blood of the 3 year old hens. These data show that the blood was very low in fat, giving an average of only 0.171 per cent. There is a marked difference between the amount of fat found in this group and that found in the blood of 1 year old hens. None of the 3 year old birds had laid, on the average, for 60 days at the time the blood samples were taken. This fact we believe warrants the conclusion that a low fat content signifies that the hen is not laying.

Of the eighty-two hens there were, then, only seventeen that were laying when the blood samples were taken; the average number of eggs from these hens was 163.7, showing a much higher average egg production than for the birds that were not laying

TABLE II.

Fat in the Blood of Twelve Hens That Had About Completed Their 3rd Year of Laying, and Days since Their Last Egg Was Laid.

No.	Fat.	Days since last egg was laid.
	per cent	
107	0.066	56
425	0.100	46
76	0.126	73
382	0.157	36
43 RR	0.161	92
401	0.166	51
430	0.168	63
386	0.176	35
102	0.194	61
404	0.208	66
116	0.228	79
110	0.297	62
Total................	2.047	720
Mean................	0.171	60

TABLE III.

Laying Hens.

No.	Yearly total.	Fat.
		per cent
69	132	0.246
15B–32	158	0.358
11	168	0.406
23	135	0.448
5L–63	147	0.526
15B–39	138	0.625
5L–70	176	0.735
57	144	1.235
15B– 5	227	1.315
89	186	1.319
5L–65	142	1.388
15B–90	211	1.641
5L–59	107	1.779
15B–57	172	1.864
43	179	1.915
12	192	1.953
Total................	2,615	17.753
Mean................	162.8	1.109

TABLE IV.

Non-Laying Hens.

No.	Yearly total.	Fat.	Days since last egg was laid.
		per cent	
15B- 9	178	0.083	61
15	120	0.085	66
13	202	0.088	28
54	113	0.114	110
16	139	0.123	66
5L-54	148	0.123	36
15B-56	124	0.124	20
18	186	0.125	9
3	168	0.131	78
60	153	0.131	22
74	165	0.133	28
49	220	0.138	10
5L-53	144	0.144	65
15B-51	154	0.154	6
19	156	0.161	61
5L-66	134	0.166	55
15B- 1	166	0.167	60
83	102	0.167	58
5L-52	147	0.167	23
15B-96	146	0.169	17
5L-55	134	0.175	22
15B-38	156	0.178	36
99	189	0.179	6
105	101	0.180	35
52	10	0.180	212
46	110	0.181	83
5L-71	140	0.183	46
68	133	0.187	88
15B-55	150	0.187	22
58	198	0.188	13
20	141	0.189	26
5L-64	170	0.191	6
77	101	0.196	60
15B-80	149	0.198	8
31	113	0.202	51
36	150	0.202	39
37	167	0.204	33
66	72	0.204	82
34	171	0.205	24
25	134	0.223	38

TABLE IV—*Concluded.*

No.	Yearly total.	Fat.	Days since last egg was laid.
		per cent	
62	96	0.228	95
98	117	0.228	55
104	138	0.230	53
82	113	0.231	54
77	166	0.241	10
91	171	0.242	22
28	132	0.253	51
5L–67	91	0.272	55
5L–62	146	0.300	62
15B–97	152	0.307	43
5L–60	149	0.312	37
51	139	0.437	50
15B–64	0	0.448	365
24	147	0.541	32
Mean............	139.09	0.199	51.7

when the blood samples were taken. The average per cent of fat found in the blood was much higher for birds that were laying than for the group of birds that were not laying (Tables III and IV). This is very striking and quite agrees with the averages reported earlier.[5] One-half of the birds had over 1 per cent of fat in their blood, as shown in Table III.

Table IV shows that the per cent of fat in the blood of non-producers was very much lower than the per cent of fat in the blood of laying birds. Every bird in this group had stopped laying from 6 to 365 days previous to the time the blood samples were taken. The average egg production for this group of birds was 139.1, which is a much lower average than for the birds that were laying when the samples were taken. Not only this, but the average per cent of fat was much lower, while the average days since they had produced any eggs was 51.7. Only one individual in this group of birds had over 0.5 per cent of fat in the blood while others gave a reading as low as 0.083 per cent.

Table V shows that if all the birds having over 1 per cent of fat in their blood are grouped together the average egg production is found to be 173.4, or an average of over 55 eggs more than that of the birds that had yellow beaks, legs, and vents,

TABLE V.

Data for Hens with Over 1 per Cent of Fat in the Blood, and with Pale Beaks, Legs, and Vents.

No.	Yearly total.	Fat.	Days since last egg was laid.
		per cent	
5L–57	144	1.235	0
15B– 5	227	1.315	0
89	186	1.319	0
5L–65	142	1.388	0
15B–90	211	1.641	0
5L–59	108	1.779	0
57	172	1.864	0
43	179	1.915	0
12	192	1.953	0
Total..........	1,561	14.409	0
Mean..........	173.4	1.601	0

and a low fat content. Further, this group of birds were all laying when the records were taken, and higher annual egg production would of course be expected from birds that had continued to lay until October, the time the blood samples were taken.

As a rule, if a bird has been laying for some time the legs, beak, and vent undergo a physiological change, and where they continue to lay, these parts will become decidedly pale in color. Table VI shows that what is true of the external parts of the body is quite true of the blood. That is, the blood of birds having external parts that have bleached have on the average a higher per cent of fat in their blood. The fat content of the blood is correlated with egg laying activity, and there is also a slight correlation between the amount of fat in the blood and high yearly production. The amount of fat is also correlated with the fading of the external parts. There is an average of 0.816 per cent of fat in the blood of hens having pale beaks, legs, and vents. When this is compared with the data for the group of hens having these parts yellow there is found to be only an average of 0.196 per cent of fat (Tables VI and VII).

Table VII shows the reverse of Table VI in the average yearly total per hen, average per cent of fat, and average number of days since the last egg was laid. The average production was only 117.9 for the birds having yellow legs, beaks, and vents,

TABLE VI.

Data for Hens with Pale Beaks, Legs, and Vents.

No.	Yearly total.	Fat.	Days since last egg was laid.
		per cent	
15B–60	153	0.131	22
51	154	0.154	6
99	189	0.179	6
105	101	0.180	35
5L–64	170	0.191	6
15B–77	166	0.241	10
32	158	0.258	0
11	168	0.406	0
23	135	0.448	0
5L–63	147	0.526	0
70	176	0.735	0
15B–5	227	1.315	0
89	186	1.319	0
5L–57	144	1.235	0
15B–90	211	1.641	0
57	172	1.864	0
43	179	1.915	0
12	192	1.953	0
Total..........	3,028	14.691	85
Mean..........	168.20	0.816	4.72

whereas the average production for the birds that were pale in those parts was 168.2. The per cent of fat found in this group of birds was only 0.196, whereas in the group of birds having pale parts it was 0.816. The average number of days since the last eggs were laid for the group of birds having pale parts was 4.722, while for the group of birds in Table VII it was 75.21. This would indicate that if a bird had yellow legs, yellow beak, and a yellow vent, the per cent of fat in the blood would be low.

It has been reported by Matthews that in man and animals the blood is much richer in fat after they have been eating than it is after fasting. This is not true with hens, as shown from Table VIII; the average per cent of fat for the birds that had been without food is a little higher than those that had not. The difference is not great, however, 0.009 per cent, but would indicate that fasting for 16 hours has no effect upon the amount of fat in the blood.

TABLE VII.

Data for Hens with Yellow Beaks, Legs, and Vents.

No.	Yearly total.	Fat.	Days since last egg was laid.
		per cent	
107x	110	0.066	56
15B– 9	178	0.083	61
15	120	0.085	66
425x	120	0.100	46
15B–54	113	0.114	110
16	139	0.123	66
5L–54	148	0.123	36
76x	111	0.126	73
15B– 3	168	0.131	78
5L–53	144	0.144	65
15B–19	156	0.161	61
401x	93	0.166	51
15B– 1	166	0.167	60
430x	107	0.168	63
15B–52	10	0.180	212
46	110	0.181	83
5L–71	140	0.183	46
15B–55	150	0.187	22
102x	113	0.194	61
5L–77	101	0.195	60
15B–31	113	0.202	51
404x	101	0.208	66
116x	83	0.228	79
15B–98	117	0.228	55
62	96	0.228	95
82	113	0.231	54
5L–67	91	0.272	55
110x	128	0.297	62
5L–62	146	0.300	62
60	149	0.312	37
51	139	0.437	50
15B–64	0	0.448	365
Total..........	3,773	6.268	2,407
Mean..........	117.906	0.196	75.21

Table IX shows the per cent of fat found in the blood of male birds. The figures for fat from these few birds indicate that the per cent of fat in the blood of male birds was more constant than in hens. This may be accounted for by the fact that the beaks,

TABLE VIII.

Fat in the Blood of Hens Fasted for 16 Hours and Hens Not Fasted.

Fasted.	Not fasted.
per cent	*per cent*
0.133	0.164
0.154	0.187
0.178	0.188
0.179	0.198
0.202	0.228
0.223	0.231
0.228	0.241
0.230	0.242
0.253	0.258
0.541	0.307
0.625	0.448
1.915	1.864
4.861	4.753
Average......0.405	0.396

TABLE IX.

Fat in the Blood of Twelve Male Birds.

No.	Fat.
	per cent
108	0.097
104	0.141
99	0.146
96	0.149
7	0.160
98	0.166
65	0.170
0	0.191
B-78	0.194
20	0.200
33	0.246
27	0.249
Total...........................	2.109
Mean...........................	0.176

legs, and ear lobes of male birds do not change in color and that there is no building of eggs within the body cavity. The reserve fat stored in the body is not used up in the same way that the female uses hers, and consequently there is no cause for the fad-

ing in the parts mentioned above. The average per cent of fat in a male bird is 0.176, or about the same as the per cent found in the blood of 3 year old hens. The blood of Bird 108 was very low in fat. This is suggestive and leads to the investigation whether the male birds that are prepotent can be detected by the fat content of their blood. (By prepotent we mean a male bird which transmits to its female offspring high fecundity.)

TABLE X.

Fat and Cholesterol in the Blood of a Few Laying and a Few Non-Laying Hens, and Also a Few Roosters.

No.	Cholesterol.	Fat.
	Laying hens.	
	per cent	*per cent*
26	0.019	1.915
9	0.047	0.526
32	0.076	0.258
28	0.082	1.864
61	0.110	1.388
76	0.114	0.246
16	0.121	1.953
64	0.149	1.235
34	0.155	0.448
62	0.169	1.779
5	0.214	1.315
Mean................	0.114	1.175
	Non-laying hens.	
41	0.023	0.138
35	0.077	0.241
60	0.078	0.180
39	0.081	0.124
68	0.106	0.191
43	0.118	0.448
79	0.121	0.735
Mean................	0.086	0.294
	Male birds.	
95	0.069	0.097
96	0.075	0.166
94	0.089	0.170
93	0.110	0.146
Mean................	0.086	0.145

SUMMARY.

The present paper is a contribution to the general problem of the relation between the amount of fat and fecundity.

The data comprise the per cent fat valuations and the egg records for eighty-two hens.

The egg records cover a period from November to October inclusive. Blood samples were taken from October 28 to November 3, 1916.

There is little or no correlation between the amount of fat in the hen's blood and her yearly egg yield. On the other hand the blood of a hen laying at the time the sample is taken is much richer in fat than that of a hen that is not laying.

The average per cent of fat found in the seventy hens that had just completed their 1st year of laying was 0.407; for the twelve old hens having just completed their 3rd year of laying the per cent was 0.171; for the twelve $1\frac{1}{2}$ year old male birds it was 0.176.

From the data at hand it seems improbable that high producing hens can be selected merely by sampling their blood and analyzing for fat unless one is careful to take into account whether or not a hen is laying at the time of sampling, and also the season of the year.

Depriving hens of food for 16 hours did not seem to lessen the per cent of fat in their blood.

There exists a close correlation between the color of the beak, legs, and vent and the per cent of fat found in the blood. The birds that had pale legs, pale beaks, and pale ani carried a very high per cent of fat in their blood and also had a high average egg production. The birds that had distinctly yellow legs, beaks, and ani gave a very low average egg production, and the per cent of fat carried in their blood was very low. This would show that birds that were not laying were storing fat in the body cells, and consequently their legs, beaks, and ani would become yellow, the natural color for all American breeds and the Leghorns.

The average per cent of fat found in the blood of the 3 year old hens was much lower than that found in the blood of the 1 year old birds. The per cent of fat found in the blood of male birds was more constant than that of 1 year old hens. There

did not appear to be very much difference between the per cent of fat found in the blood of male birds and that found in the blood of females not laying.

It seems feasible to believe that the principal reason why the blood of laying hens is much richer in fat than that found in birds not laying is that the fat stored in the body tissues is taken up by the blood and carried to the egg yolk.

TRITICO NUCLEIC ACID.

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Recent investigations have definitely indicated that there are two distinct types of nucleic acid, one of which is found in animal tissues and the other in plants. While this distinction rests upon the examination of a large number of nucleic acids of animal origin, our knowledge of plant nucleic acid has been acquired from a study of but two substances; one of these is prepared from yeast (yeast nucleic acid) and the other from the wheat embryo (tritico nucleic acid).

In 1902 Osborne and Harris[1] prepared tritico nucleic acid and showed that like all other nucleic acids it produces phosphoric acid, guanine, and adenine by hydrolysis with dilute mineral acids. But tritico nucleic acid was found to differ from all animal nucleic acids and to resemble yeast nucleic acid by its production of uracil instead of thymine, and pentose instead of levulinic acid. At the time Osborne and Harris' paper appeared cytosine was not known, but Wheeler and Johnson[2] afterwards found cytosine among the hydrolytic products of tritico nucleic acid.

So far as concerns their fundamental groups, therefore, the two plant nucleic acids differ sharply from all animal nucleic acids and are identical with one another.

After Levene and Jacobs[3] had shown that by hydrolysis with ammonia at high temperatures yeast nucleic acid loses its phosphoric acid and produces four nucleosides, it became of great interest to know whether tritico nucleic acid would behave in the

[1] Osborne, T. B., and Harris, I. F., *Z. physiol. Chem.*, 1902, xxxvi, 85.

[2] Wheeler, H. L., and Johnson, T. B., *Am. Chem. J.*, 1903, xxix, 505.

[3] Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, 1910, xliii, 3150.

same manner. Levene and La Forge[4] found this to be the case. They described a very efficient method of preparing tritico nucleic acid and found that the substance yields three of the four nucleosides of yeast nucleic acid (guanosine, adenosine, and cytidine). They made no attempt to find uridine.

Hence, not only in their fundamental groups but in the formation of nucleosides the two plant nucleic acids are seen to be identical so far as experiments have been made, and we shall extend the coincidence to include uridine.

It has very recently been shown in this laboratory[5] that when yeast nucleic acid is hydrolyzed with ammonia at *low temperatures* no phosphoric acid is removed and two nucleotides are formed; *viz.*, guanine mononucleotide and adenine-uracil dinucleotide.

In the following we show that these same nucleotides are produced by tritico nucleic acid. The guanine mononucleotide obtained from tritico nucleic acid does not differ either in its chemical composition or in any of its chemical properties from the substance formerly prepared from yeast nucleic acid. By mild acid hydrolysis it produces guanine but not adenine, and sets free its entire phosphoric acid. By treatment with brucine it forms a crystalline brucine salt which melts at 203°C.

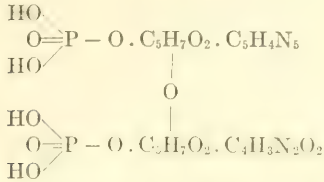
Adenine-uracil dinucleotide prepared from tritico nucleic acid is identical with that prepared from yeast nucleic acid. By mild acid hydrolysis it produces adenine but not guanine, and liberates only half of its phosphoric acid. By hydrolysis with ammonia at high temperatures it produces both adenosine and uridine. It forms with brucine a crystalline brucine salt which has the composition required for the formula $C_{19}H_{25}N_7O_{15}P_2(C_{23}H_{26}N_2O_4)_4.14H_2O$, and melts at 175°C.

The nitrogen percentage of the brucine salt shows that it contains a uracil group, not a cytosine group, and that uracil and uridine are not obtained from tritico nucleic acid as secondary products to cytosine and cytidine. The presence of four brucine radicles in the brucine salt leads to the disaccharide structure of the dinucleotide.[6]

[4] Levene, P. A., and La Forge, F. B., *Ber. chem. Ges.*, 1910, xliii, 3164.

[5] Jones, W., and Read, B. E., *J. Biol. Chem.*, 1917, xxix, 111. Read, B. E., *ibid.*, 1917, xxxi, 47.

[6] For argument see Jones and Read, *J. Biol. Chem.*, 1917, xxix, 113.



Although it is not possible to prove rigidly the identity of two substances the exact coincidence in so many properties of tritico nucleic acid with yeast nucleic acid leaves little room to doubt that they are the same chemical compound.

EXPERIMENTAL.

100 gm. of tritico nucleic acid prepared from the wheat embryo by the method of Levene and La Forge[4] were dissolved in 530 cc. of 2.5 per cent ammonia, and the solution was heated in an autoclave for 1½ hours at 115°. The cooled product was treated with 530 cc. of absolute alcohol when the ammonium salt of guanine mononucleotide was precipitated, leaving the ammonium salt of adenine-uracil dinucleotide in solution. The two substances were separated by filtration, purified, and the two nucleotides finally obtained as white powders by the methods already described in connection with yeast nucleic acid.[5]

Guanine Mononucleotide.—Commercial yeast nucleic acid evidently contains various impurities which contaminate products that are prepared from it. This is not the case with tritico nucleic acid which produces guanine mononucleotide so free from foreign substances that its purification offers little difficulty. The substance which we obtained gave the following analytical data.

I. 0.2634 gm. dried at 100° required 13.65 cc. H_2SO_4 (1 cc. = 0.0037 gm. N).
II. 0.2227 " " " " " 11.50 " " (1 " = 0.0037 " ").
III. 0.4566 " " " " gave 0.1415 gm. $Mg_2P_2O_7$.
IV. 0.4882 " " " " " 0.1525 " "

	N	P
Calculated	19.28	8.54
Found.		
I	19.17	
II	19.11	
III		8.65
IV		8.72

Hydrolysis of Guanine Mononucleotide with Dilute Mineral Acid. —Portions of the guanine mononucleotide were heated with twenty volumes of 5 per cent sulfuric acid and estimations of phosphoric acid and guanine were made with the product.

I.	0.3487 gm.	dried at	100°	and hydrolyzed	for	3 hrs.	gave	0.1069 gm.	$Mg_2P_2O_7$.
II.	0.3394 "	"	" 100°	"	"	" 3 "	"	0.1032 "	"
III.	0.4924 "	"	" 100°	"	"	" 1 hr.	"	0.1806 "	guanine.
IV.	0.7170 "	"	" 100°	"	"	" 1 "	"	0.2677 "	"

	P	Guanine.
Calculated	8.54	41.6
Found.		
I	8.57	
II	8.49	
III		36.68
IV		37.30

These results show that the substance loses its entire phosphoric acid by mild acid hydrolysis and therefore contains no pyrimidine group.

The final filtrates from magnesium ammonium phosphate gave no gelatinous precipitate of silver-adenine upon treatment with ammoniacal silver solution.

The Brucine Salt of Guanine Mononucleotide.—A solution of 1 gm. of guanine mononucleotide in 5 cc. of hot water was treated with 2.5 gm. of brucine in 5 cc. of hot absolute alcohol. The crystalline dibrucine salt was immediately deposited in characteristic rosettes, which after washing with hot absolute alcohol and recrystallization from hot water melted at 203°C.

Preparation of Guanosine from Guanine Mononucleotide.—Guanine mononucleotide was heated with five parts of 2.5 per cent ammonia in an autoclave for 2 hours at 140°C. On cooling in the ice chest guanosine was deposited as a gelatinous mass. This was filtered on a Buchner funnel and recrystallized from hot water with the use of animal charcoal. Pure guanosine was obtained in long transparent needles which on analysis gave the following results.

I. 0.2382 gm. air-dried substance required 14.11 cc. H_2SO_4 (1 cc. = 0.0037 gm. N).
II. 0.8086 gm. heated at 120°C. lost 0.0905 gm.

	H_2O	N
Calculated	11.29	21.94
Found.		
I		21.92
II	11.19	

Adenine-Uracil Dinucleotide.—Specimens of adenine-uracil dinucleotide prepared as described were submitted to hydrolysis with 5 per cent sulfuric acid. The product gave no precipitate of guanine on the addition of ammonia. The solution was treated directly with magnesia mixture and the free phosphoric acid determined as magnesium ammonium phosphate. The final filtrate from magnesium ammonium phosphate gave the characteristic gelatinous silver-adenine precipitate upon treatment with ammoniacal silver solution; so that the particular structure of the compound as a dinucleotide composed of one pyrimidine group containing firmly bound phosphoric acid and one purine group containing a phosphoric acid radicle easily set free by hydrolysis with dilute mineral acid is thus fully established.[7] Analyses for total and partial phosphorus gave results consistent with this structure.

I. 0.6936 gm. dried in a desiccator gave 0.2442 gm. $MgNH_4PO_4.6H_2O$.
II. 0.7803 " " " " " " 0.2751 " "
III. 1.2024 " " " " " " after complete destruction of organic matter 0.7762 gm. $MgNH_4PO_4.6H_2O$.

	I.	II.	III.
Substance used	0.6936	0.7803	1.2024
Time of hydrolysis	3 hrs.	3 hrs.	Total.
$MgNH_4PO_4.6H_2O$ obtained	0.2442	0.2751	0.7762
Amount calculated per gm	0.3521	0.3535	0.6450
Pyrimidine correction	0.0300	0.0300	
From purine nucleotide	0.3221	0.3235	
Half the total phosphorus			0.3225

[7] For full discussion of this subject see Germann, H. C., *J. Biol. Chem.*, 1916, xxv, 189.

The above results show that exactly half of the phosphoric acid of this dinucleotide is easily split and the other half is firmly bound.

Preparation of Uridine from the Dinucleotide.—25 gm. of dinucleotide were dissolved in 125 cc. of 2.5 per cent ammonia and heated in an autoclave at 133°C. for 2 hours. The product was allowed to stand in ice water for several hours but no trace of guanosine was deposited.

A portion of the autoclave product was warmed and treated with a hot aqueous solution of picric acid until cold picric acid failed to give a precipitate with a drop of the fluid. Upon recrystallization of the bulky gelatinous precipitate from hot water, adenosine picrate was obtained in characteristic transparent yellow plates.

The main portion of the autoclave product was treated for uridine by the method of Levene and La Forge.[4] Snow-white crystalline uridine was obtained, which melted at 158° (corrected), and gave the following analysis.

0.3746 gm. air-dried substance required 11.57 cc. H_2SO_4 (1 cc. = 0.0037 gm. N).

	N
Calculated	11.48
Found	11.43

Brucine Salt of the Dinucleotide.—3 gm. of substance dissolved in 9 cc. of hot water were treated with a solution of 7.2 gm. of brucine in 15 cc. of hot absolute alcohol. After washing the precipitated brucine salt thoroughly with hot alcohol, it was recrystallized from fifty parts of hot water. The characteristic crystalline substance melted at 175°, and gave the following analytical data.

I. 0.2222 gm. heated at 115°C. lost 0.0228 gm.
II. 0.4300 " gave 33.2 cc. N at 757 mm. and 28°C.
III. 0.4458 " " 34.6 " " " 754 " " 27°C.
IV. 1.2110 " " on complete oxidation of all organic matter 0.1098 gm. $Mg_2P_2O_7$.
V. 1.3013 gm. gave on complete oxidation of all organic matter 0.1189 gm. $Mg_2P_2O_7$.
VI. 0.3867 gm. gave 0.2750 gm. crystalline brucine, $C_{23}H_{26}N_2O_4.4H_2O$ (no chloroform extraction was made of the mother liquor).

	H_2O	N	P	Brucine.
Calculated	10.16	8.46	2.49	75.1
Found.				
I	10.26			
II		8.44		
III		8.47		
IV			2.49	
V			2.55	
VI				71.1

THE PROTEOCLASTIC TISSUE ENZYMES OF THE SPLEEN.

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Problem.—Two autolytic enzymes have been described in the spleen. The one is described (1–4) as being active in an alkaline medium, adsorbable by kieselguhr and extractible with 5 per cent NaCl solution. This is the α-protease of Hedin and his coworkers. The other enzyme which is capable of hydrolyzing proteins is the β-protease of these investigators, having the characteristics of being salted out with ammonium sulfate and of being extractible with dilute acetic acid. It is not adsorbed by kieselguhr.

The writer has attempted to determine the range of activity of autolyzing enzymes, and inasmuch as it appears that the spleen varies from other tissues in regard to the relation between enzyme activity and hydrogen ion concentration (5), a study has been made concerning this relation in spleen enzymes.

Methods.

Spleens from the ox and the dog were used; the former, obtained from the hospital butcher, was about 48 hours old; the latter was prepared from a freshly killed dog. No characteristic differences in spleen enzymes were observed in the two cases.

The digests were prepared in the same manner in which they have been prepared in the former studies on tissue enzyme action (6). The tunica was removed before the spleen pulp was ground. Suspensions were made in the various phosphate mixtures given below so that 40 gm. net weight of spleen tissue were made up to 100 gm. by means of the phosphate mixtures. These mixtures were prepared from Sörensen's chart (7), the pH varying from 5.68 to 7.59 for beef spleen and to 7.98 for that of the dog. These mixtures were then read on a Leeds and Northrup Type K potentiometer. A 0.1 N calomel electrode, Leeds and Northrup standard cadmium element, and a Clark (8) oscillating hydrogen vessel with platinum electrode were

used. The hydrogen was obtained electrolytically and purified by means of a palladium sponge filter of de Khotinsky.[1]

α-Amino nitrogen determinations were made by means of the gasometric method of Van Slyke. Aliquots were used from the filtrates of 2.5 per cent trichloroacetic acid precipitates for these determinations.

Reaction.	Amino nitrogen per 40 gm. of fresh tissue.		
	Initial.	24 hrs.	6 days.
pH	*gm.*	*gm.*	*gm.*
5.68	0.221*		0.343
6.55	0.221		0.322
6.58	0.221		0.290
6.80	0.221		0.268
6.94	0.221		0.238
7.08	0.221		0.238
7.59	0.221		0.211
7.98	0.0053**	0.0053	0.0055

* Beef.

** Dog.

DISCUSSION.

It will be observed that digestion occurs where pH $<$ 7.08. pH 7.07 is approximate neutrality at the temperatures at which these readings were made. A departure from neutrality towards alkalinity results in inhibition of hydrolysis *as far as the proteins of the tissue are concerned.* Apparently the enzyme or enzymes are active at an alkalinity of pH 7.5–7.9, as will be seen from the following experiment where 5 gm. of Witte's peptone were introduced into a digest composed as the one given above for dog spleen in pH 7.98 phosphate mixture.

	Reaction.	Amino nitrogen per 40 gm. of tissue.	
		Initial.	24 hrs.
	pH	*gm.*	*gm.*
Control..........................	7.98	0.054	0.054
" + 5 gm. of peptone.......	7.98	0.107	0.217

[1] Through the generosity of Mr. Albert Kuppenheimer of Chicago the Institute has been able to procure physicochemical apparatus of various sorts. The present work was done by means of the apparatus for electromotive force, purchased from this fund.

The peptone is digested to a marked extent, showing that a protein-hydrolyzing enzyme (or enzymes) is active. It is difficult to explain, however, why, if the enzymes are active at neutrality and in an alkaline reaction, no increase in α-amino nitrogen is obtained from the polypeptides which occur in partly autolyzed tissue, such as that obtained 48 hours after the animal's death; for the writer has determined that peptones and higher amino-acid linkages occur to a considerable extent in liver from a meat market. On adding large amounts of peptone and albumose, as in these experiments, a correspondingly large increase in amino nitrogen is obtained over that which is present initially. The conclusion is unescapable that native peptones and other polypeptides are not hydrolyzed by the tissue enzymes under the conditions of these experiments, although those introduced are digested.

Inasmuch as these experiments show that as far as the native proteins of the spleen are concerned, no alkaline-acting enzyme is recognizable capable of hydrolyzing these proteins, the experiments are divergent from those of Hedin and others who have described an enzyme of this nature, the so called α-protease. The question arises as to whether the α-protease is a true autolytic enzyme, belonging to the spleen tissue, or whether it is not of the nature of Martin Jacoby's *heterolytic* enzyme, and is derived from the leukocytes which abound in the spleen. For these bodies, alkaline-acting enzymes have been described by Opie (9) and later by Jobling and Strouse (10). Leukoproteases have been described by Leber (11), Jochmann and Ziegler (12), and others, following the initial work of Müller (13), but the range of their activity in regard to reaction of medium is not known.

CONCLUSIONS.

An enzyme or enzymes exist in the spleen, capable of hydrolyzing peptone and also (as shown by Hedin) fibrin. The proteins of the spleen itself, however, autolyze only in neutral or acid, not in alkaline solution. The most rapid autolysis was observed in the most acid solution tested, pH = 5.68.

From the standpoint of necrosis, the α-protease of Hedin can scarcely be operative since there is no evidence that it affects native proteins.

It is probable that the α-protease of Hedin is not an autolytic enzyme, but rather a heterolytic one, resident in the white blood cells, for such an enzyme has been described, definitely, for such cells.

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COMPARATIVE METABOLISM OF CERTAIN AROMATIC ACIDS.

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Fate of Phenylacetic Acid in the Organism of the Monkey.

Many experiments have been reported showing a marked difference between metabolism in the human body and in the lower animals. This alone has stimulated considerable interest on account of the apparent lack of reason for such difference. In the last few years experiments have been carried out with animals more closely related to the human in order to compare their metabolism with that of both human beings and lower animals.

Hunter and Givens (1) report the purine metabolism in the monkey as resembling that of the lower animals and not the human. In the urine of the monkey (*Cercopithecus*) allantoin accounted for 73 per cent of the nitrogen arising from the catabolism of the endogenous purines while the remainder appeared principally as purine bases and none as uric acid. Later work by the same authors (2) shows like results for another monkey (*Cercopithecus callitrichus*). Wiechowski (3) obtained results comparable to those of Hunter and Givens but was unable to find allantoin as the end-product of the purine metabolism of the chimpanzee. Baumann and Oviatt (4) found the urinary sulfur excretion in the monkey (*Macacus*) quite different from that of man. In the case of the monkey the ratio of inorganic to ethereal sulfates was approximately $3\frac{1}{2}$ to 1 while for man it bore the ratio 12 to 1.

An interesting phase of this same question is the manner in which the different animal organisms detoxicate certain protein putrefactive products. The *series of compounds* resulting from the action of putrefactive bacteria on the aromatic amino-acids must be detoxicated and eliminated from the animal body as

soon as possible. This detoxication process seems often to differ for different species or at least results in the excretion of an entirely different end-product for the acid thus resorbed.

Phenylacetic acid is one of those aromatic acids which yield different conjugation compounds in the urine after ingestion by men and animals respectively. Phenylacetic acid in combination with glycocoll is excreted normally by many of the herbivora as phenaceturic acid. Phenaceturic acid was discovered by Salkowski (5) in normal horse urine. Phenaceturic acid has been found in the urine of many different animals after feeding phenylacetic acid. Thus E. and H. Salkowski (6) isolated it from the urine of dogs and rabbits, and Vasiliu (7) from the urine of sheep after phenylacetic acid feeding. E. Salkowski (8) believed he had found traces of the phenaceturic acid in normal human urine. Totani (9) isolated from the excreta of chickens phenylacetylornithuric acid after phenylacetic acid feeding. Thierfelder and Sherwin (10) found in human urine two compounds, phenylacetyl glutamine and phenylacetyl glutamine urea after various amounts of phenylacetic acid had been ingested but were unable to find even a trace of either phenaceturic acid or uncombined phenylacetic acid.

Before beginning certain metabolism work on a monkey it was important to know whether the fate of the phenylacetic acid in the organism of the monkey would resemble that of the human organism or that of the lower animals. The combination with glycocoll resulted showing the metabolic process to resemble that of the lower animals, not only in the case of the phenylacetic but also for the *p*-hydroxyphenylacetic and *p*-hydroxy benzoic acid (11).

EXPERIMENTAL.

A 4.2 kg. female monkey (*Macacus rhesus*) was placed on a regular diet of milk, bread, bananas, and apples for several days in order to see if the urine contained traces of any phenylacetic acid compound. After no compound of this nature could be found in the urine, the monkey was fed 1 gm. of phenylacetic acid per day as the soluble sodium salt. The sodium salt dissolved in fresh milk at first seemed quite acceptable, but after the 3rd day it was refused. Any food or liquid possessing even the slightest odor of phenylacetic acid was entirely refused by the monkey. At this point forced feeding was resorted to and the water solution of the sodium salt introduced directly into the stomach by means of a stomach tube. The

amount thus given varied between 1 and 2 gm. The physiological effect seemed to be much the same as that produced on human beings. Each dose was followed by a period of dullness and inactivity, while the ingestion of 2 gm. (0.47 gm. per kg. body weight) resulted in an entire loss of appetite for 24 hours. When the phenylacetic acid is ingested by men in quantities varying from 0.05 to 0.26 gm. per kg. body weight intoxication results much the same as that produced by alcohol.

The monkey's urine was collected for 36 hours after each dose of the acid, and the different portions were united and acidified with phosphoric acid until a distinct acid test with Congo red resulted. The concentrated urine was then extracted repeatedly in a liquid extracting apparatus with ethylacetate until no phenylacetic acid compound formed by evaporation of the last extraction. The ethylacetate extract thus prepared was evaporated to one-half its original volume and placed on ice for 24 hours. As no phenylacetyl glutamine crystallized out at this concentration, the extract was again evaporated and the evaporation continued on subsequent days until the entire ethylacetate solution amounted to about 100 cc. only. At this concentration, there being no possibility of obtaining any phenylacetyl glutamine, the extract was evaporated carefully almost to dryness and then taken up with 50 cc. of water and boiled with charcoal to remove the pigment. After filtering this water solution from the charcoal, evaporating, and allowing the concentrated solution to stand on ice, there appeared small rhombic crystals of phenaceturic acid. The mother liquor from these crystals was optically inactive, showing again the absence of phenylacetyl glutamine. After the feeding of 1 gm. of phenylacetic acid the amount of phenaceturic acid isolated from the urine amounted to only 0.82 gm. or 51 per cent of the expected yield.

The phenaceturic acid was twice recrystallized from water, dried *in vacuo*, and then used for analyses.

Melting point 142–143°.

0.1232 gm. of the acid required 6.29 cc. of 0.1 N sodium hydroxide instead of 6.37 cc., the theoretical amount.

0.1331 gm. substance gave 8.50 cc. nitrogen at 21° and 748 mm. pressure.

	Calculated for $C_{10}H_{11}NO_3$:	Found:
N	7.25	7.15

CONCLUSION.

The metabolism of phenylacetic acid in the organism of the monkey is the same as that found in the lower animals and entirely different from the metabolism of the same substance in man.

Phenylacetic acid in the monkey is conjugated with glycocoll and excreted as phenaceturic acid, while in man it is conjugated with glutamine and excreted partly as phenylacetyl glutamine and partly as phenylacetyl glutamine urea.

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STUDIES ON BIOLUMINESCENCE.

VIII. THE MECHANISM OF THE PRODUCTION OF LIGHT DURING THE OXIDATION OF PYROGALLOL.

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INTRODUCTION.

It has been very definitely established from the observations of many investigators that the production of light by animals is a chemiluminescent phenomenon; *i.e.*, a luminescence accompanying a chemical reaction. As free oxygen is necessary for light production in all forms, the reaction may be classed among the oxidations. In several animals two substances (Harvey, 1) in addition to water and oxygen are necessary to produce light and at least one of these is oxidized. As a number of compounds of definite chemical composition are known which emit light during oxidation, it seemed desirable to study such reactions more in detail in order to determine under what conditions the light is emitted.

In a previous paper (Harvey, 1916) I showed that pyrogallol in weak solutions would produce light if oxidized by the plant peroxidases or by blood containing hemoglobin or by certain salts ($KMnO_4$, $K_4Fe(CN)_6$, and $FeCl_3$) if H_2O_2 were present. These substances may be collectively spoken of as oxidizers. No light appeared in the absence of H_2O_2 and no light appeared with pyrogallol and H_2O_2 alone. All means of oxidizing pyrogallol with light production were also efficacious in blueing gum guaiac, but guaiac can be oxidized by many methods which fail to produce light with pyrogallol. The oxidation of gum guaiac was never observed to produce light nor was light ever observed during the oxidation by peroxidases of many other easily oxidizable hydroxy-phenyl and amino-phenyl compounds. No other peroxide was found that could take the place of H_2O_2 in the luminous oxidation of pyrogallol by plant peroxidases. The peroxidase unquestionably acts by transferring oxygen from H_2O_2 to the pyrogallol, and both H_2O_2 and peroxidase are used up in the process (2). It is not necessary that H_2O_2

be decomposed with formation of bubbles of oxygen. KCN, acids, alkalies, and heat affect the light production in a characteristic way because they affect the peroxidase. It is consequently necessary, in order to gain a more accurate knowledge of the conditions under which the oxidation of pyrogallol leads to light production, to employ oxidizing substances of definite composition which are not affected by temperature, acid, etc. The present paper deals with the production of light by these inorganic substances.

The first observation that pyrogallol would luminesce was made in connection with photography. Lenard and Wolf (6) noticed that a photographic plate which was developed with a pyrogallol—Na_2SO_3—K_2CO_3 developer glowed, if dipped in a saturated alum solution. They found also that the developer itself would light on mixing with alum solution, and thought that light was due to pyrogallol, which collected about the $Al(OH)_3$ precipitated by K_2CO_3 and was there oxidized. $K_2S_2O_5$ might replace the Na_2SO_3 of the developer (21).

Trautz and Schorigin (8) also observed the luminescence of a photographic plate as described by Lenard and Wolf and noted in addition that 30 per cent formaldehyde might take the place of the saturated alum solution. In addition they noticed that a piece of raw leather, extracted with ligroin to remove fat, will luminesce if moistened with 30 per cent formaldehyde and exposed to the air. Further work showed that both aldehydes and phenol derivatives luminesce when oxidized in many ways, as follows.

Aldehydes.

1. With (*a*) neutral or (*b*) alkaline H_2O_2.

(*a*) CH_2O (35 per cent) + H_2O_2 (3 to 30 per cent) = $CO_2 + H_2O + H_2$.

(*b*) $2CH_2O + 2NaOH + H_2O_2 = 2\ HCOONa + 2\ H_2O + H_2$.

(Acetic, propionic, valeric, benzoic, and salicylic aldehydes and glucose give light under the same conditions.)

2. With Na_2O_2 in ice water (formaldehyde, acetaldehyde, glucose, and vanillin).

3. With a warm saturated solution of $NaClO_3$ (formaldehyde).

4. With alkalies (simple aldehydes, sugars, alcohols, and many other substances).

Phenols.

1. With warm neutral H_2O_2 or alkaline H_2O_2 at room temperatures (pyrogallol, tannic, and gallic acids, benzkatechin, metol, eikonogen, and naphthol).

2. With alkalies (pyrogallol, tannic, and gallic acids and many others).

According to Trautz and Schorigin, when 2 cc. of 10 per cent pyrogallol are mixed with 5 cc. of 10 per cent Na_2CO_3, the solution glows at the surface in contact with air; if some formaldehyde is added to this, the light lasts a long time, and if H_2O_2 is also added the light is much brighter. Trautz' well known luminescent reaction with pyrogallol results when 35 cc. of 50 per cent K_2CO_3, 35 cc. of 10 per cent pyrogallol, 35 cc. of 35 per cent

formaldehyde, and 50 cc. of 30 per cent H_2O_2 are mixed. A reddish glow appears, whose tint is no doubt due to the rapidly formed dark brown oxidation products of the pyrogallol. It is not necessary to use concentrated H_2O_2, as I have obtained a beautiful glow with 2 cc. of pyrogallol (12.5 per cent), 5 cc. of 10 per cent Na_2CO_3, 2 cc. of 40 per cent formaldehyde, and 1 cc. of 3 per cent H_2O_2. When the luminescence has faded, light again appears if more 3 per cent H_2O_2 is added. In my experience, Na_2CO_3 and pyrogallol alone, or pyrogallol + Na_2CO_3 + 3 per cent H_2O_2, or pyrogallol + formaldehyde, or pyrogallol + formaldehyde + 3 per cent H_2O_2, or pyrogallol + 3 per cent H_2O_2 gave no light. As we shall see, the proportions of substances and temperature greatly influence the light and my failure to observe light with pyrogallol and Na_2CO_3 alone may possibly be explained on this basis.

Trautz and Schorigin found also that hydroquinone and resorcinol, but not phenol would luminesce under the same conditions as pyrogallol. The light was stronger the greater the concentration of (1) the pyrogallol, (2) aldehyde, (3) alkali, or (4) H_2O_2, and (5) the higher the temperature. To give the brightest light the relation between aldehyde and pyrogallol should be: CH_2O : pyrogallol = (5–10) : 1. With the same substance, the time the light lasts is longer the weaker the light. These results suggest that reaction velocity is the factor determining the production of light, and Trautz (9) is fully convinced that such is the case. My own observations on temperature and concentrations of reacting substances to be described below do not wholly bear this out.

Many other types of substances besides aldehydes and derivatives of phenols can luminesce. Such cases have been collected and added to in the extensive paper by Trautz (9) and include among organic compounds acetylene derivatives, aldehydes, monobasic saturated primary alcohols, monobasic fatty acids, polyphenols, substances with ammonia residues, and substances with condensed benzene nucleus.

Among these are found many compounds of biological interest; *i.e.*, many products of plants or animals, as the following: terpene or fatty oils and waxes, glucose, lecithin, cholesterol, cholic, taurocholic, and glycocholic acids, cetyl and myricyl alcohol (Radziszewski, 10); benzaldehyde and vanillin, tannic and gallic acids, glycerol, mannite, papaverin (Trautz, 8, 9); uric acid and asparagine (Guinchant, 11); esculin (Dubois, 12); substances in humus (Weitlaner, 18); and substances in urine, the anaerobic alkaline hydrolysis products of glue, and Witte's peptone (McDermott, 13).

The various types of chemiluminescent reactions of general or biological interest may be grouped as below. The list also includes types described in the present paper.

1. Oxidation in air spontaneously.
   - (*a*) At ordinary temperatures. Phosphorus. Fresh cut surfaces of Na or K.
   - (*b*) At melting or vaporizing points. Fats, terpenes, sugars, resins, gums, ether, silk, and others.

2. Neutralization of strong bases and acids. CaO or BaO with concentrated H_2SO_4 or water. KOH with strong acids. $Ca(OH)_2$ and perchloric acid.
3. Decomposition of H_2O_2. If dropped on Ag_2O, Pb_3O_4, PbO_2, MnO_2, Ag, Pt, or Os.
4. Oxidation in aqueous or alcoholic alkalies. Many organic substances.
5. " " hypoiodites, hypobromites, or hypochlorites. Many organic substances.
6. Oxidation in peroxides (H_2O_2 or Na_2O_2). Many organic substances.
7. " " ozone.
8. " " acid permanganate. Pyrogallol (see p. 331).
9. " " persulfates and perborates. Formaldehyde, paraformaldehyde.
10. Oxidation in perchlorates, periodates, and perbromates. Palmitic acid.
11. Combination of 4 and 6. Many organic substances.
12. " " 5 " 6. " " "
13. " " 8 " 6. " " "
14. " " 5 " 9. " " "
15. Oxidation with H_2O_2 and hemoglobin or vegetable oxidases. Pyrogallol, lophin, esculin.
16. Oxidation with H_2O_2 and MnO_2, $Fe_2Fe(CN)_6$, $Mn(OH)_2 + Mn(OH)_3$, Ag_2O. Pyrogallol (Table I).
17. Oxidation with H_2O_2 and ferrocyanides, chromates, bichromates, permanganates, Fe salts, and Cr salts. Pyrogallol.
18. Oxidation with H_2O_2 and colloidal Ag and Pt. Pyrogallol (Table I).

In studying luminescence, Trautz and other observers have worked with concentrated solutions. It is not only unnecessary to use concentrated solutions to obtain light but concentrated solutions are to be avoided in many cases. For instance, M pyrogallol (12.6 per cent) will give no light if mixed with M/20 potassium ferrocyanide and some 3 per cent H_2O_2, but M/100 pyrogallol will give a good light, M/1,000 pyrogallol will give a brighter light, and M/100,000 pyrogallol will give a just visible light. We should expect to obtain a brighter light the greater the concentration of the pyrogallol, yet such is not the case. We should also expect to obtain a brighter light the greater the concentration of the $K_4Fe(CN)_6$ but neither is that the case. M/2 $K_4Fe(CN)_6$ will give no light with a mixture of M/100 pyrogallol and 3 per cent H_2O_2, whereas M/20 $K_4Fe(CN)_6$ does. The present paper deals with such anomalous phenomena as the above and with the mechanism of light production by pyrogallol in general. Additional substances have also been discovered which give light with

pyrogallol. The subjects treated can be conveniently considered under the following heads.

1. Oxidizers giving light with pyrogallol plus hydrogen peroxide.
2. Luminescence during electrolysis.
3. Peroxides giving light with pyrogallol and oxidizer.
4. Concentration of pyrogallol and oxidizer and light production.
5. Temperature and light production.
6. Light production in absence of peroxide.
7. Light production in non-aqueous solvents.
8. Effect of acid and alkali on light production.

EXPERIMENTAL.

1. Oxidizers Giving Light with Pyrogallol plus Hydrogen Peroxide.—Many substances in addition to those enumerated in my former paper have been found to oxidize pyrogallol with light production, but only if H_2O_2 is also present. As mentioned above, the concentration of pyrogallol has such a marked influence on light production that in the following experiments the tests were always made by mixing equal parts of M/100 pyrogallol and neutral 3 per cent H_2O_2, and adding to this an equal amount of the solution to be tested. The results are collected in Table I.

The substances tested can be divided into three groups according as they are present in true solution, in colloidal solution, or as precipitates. The concentrations given indicate those tried, in the case of substances giving no light, or the concentration giving the best light, in the case of substances which do give light. The power to blue guaiac alone or with H_2O_2 and the decomposition of H_2O_2 are also recorded.

Among the salts in solution, the ferrocyanides, chromates, bichromates, permanganates, iron and chromium compounds, and hypochlorite, hypoiodites, and hypobromites give light. Among the precipitates, $Fe_2Fe(CN)_6$, Ag_2O, and manganese dioxides, oxides, and hydroxides give light. Among the colloidal substances, in addition to the natural oxidizers in the blood of vertebrates and certain worms (hemoglobin), certain crustacea and mollusks (hemocyanin?), and plant juices (peroxidases), colloidal silver and platinum, and colloidal manganese compounds (possibly $Mn(OH)_3$) formed on the addition of $KMnO_4$ to proteins and organic substances gave light. In general, these compounds have a marked power of blueing guaiac, alone or with H_2O_2, but many

TABLE I.

Substances Giving Light with Pyrogallol and Hydrogen Peroxide.
True Solutions.

	Equal volume added to mixture of 1 part M/100 pyrogallol or 1 part 3 per cent H_2O_2 + 1 part M/100 pyrogallol; hence, concentrations in final mixture are one-half that given.		Light with pyrogallol.	Light with pyrogallol + H_2O_2	Blueing of gum guaiac	Blueing of gum guaiac + H_2O_2.	Liberation of oxygen from H_2O_2.
1	Potassium ferrocyanide	($K_4Fe(CN)_6$ M/10–M/20)	—	Bright.	+		—
2	" ferricyanide	($K_3Fe(CN)_6$ M/10–M/1,250)	—	Very faint to —.	—	—	Very slow.
3	" chromate	(K_2CrO_4 M/20–M/100)	—	Good.	+		—
4	" bichromate	($K_2Cr_2O_7$ M/50–M/100)	—	"	+		+
5	" permanganate	($KMnO_4$ M/50–M/200)	—*	Bright.	+	—**	+
6	" hydroxide	(KOH M–M/6,250)	—	—	—	—	Very slow.
7	" chlorate	($KClO_3$ M/10)	—	—	—	—	—
8	" persulfate	($K_2S_2O_8$ M/10–M/128)	—	—	—	—	—
9	" chromium alum	($Cr_2(SO_4)_3 . K_2SO_4$ M/10)	—	Faint.	Very slow.	Very slow.	—
10	Ferric ammonium alum	($Fe_2(SO_4)_3 . (NH_4)_2SO_4$ M/10)	—	"	+		Very slow.
11	" chloride	($FeCl_3$ M/10–M/250)	—	Fair.	+		Slow.
12	Ferrous sulfate	($FeSO_4$ M/10–M/6,250)	—	"	—	+	"
13	Copper sulfate	($CuSO_4$ M/5–M/125)	—	—	—	+	Very slow.
14	Chromic acid	(CrO_3 M/100)	—	Bright.	+		+
15	" sulfate	($Cr_2(SO_4)_3$ 2 per cent)	—	Faint.	—	+	Slow.
16	Manganous chloride	($MnCl_2$ M/2–M/125)	—	—	—	Slow.	"
17	" acetate	($Mn(CH_3COO)_2$)	—	—	—	—	Very slow.

18	Chlorine water		—	—	+		+
19	Bromine "		—	—	+		+
20	Iodine in KI		—	—	+		+
21	Sodium hypochlorite	(Cl water + NaOH)	Faint flash.	Bright.	+		++
22	" hypobromite	(NaOBr, bromine water + NaOH)	"	"	+		++
23	" hypoiodite	(I in KI + NaOH)	—	Faint.	+		+
24	Calcium hypochlorite	($Ca(OCl)_2$ saturated solution)	—	Good.	+		++
25	Silver nitrate	($AgNO_3$ 2 per cent)	—	—	—	—	—
26	Molybdic acid	(MoO_3 saturated solution)	—	—	—	+	Very slow.
27	Sodium molybdate	(Saturated MoO_3 neutralized with NaOH)	—	—	—	+	+
28	Uranium acetate	($UO_2(C_2H_3O_2)_2$ M/20–M/2,500)	—	—	—	—	—
	Colloidal solutions.						
1	Turnip juice		—	Bright.	—	+	++
2	" " heated to 70°		—	Faint.	—	+	Very slow.
3	" " boiled		—	—	—	—	—
4	" " + $KMnO_4$		—	Bright.	+		++
5	" " + $KMnO_4$ to 70° and filtered, filtrate		—	Faint.	—	+	—
6	" " " " " " precipitate		—	"	Slow.	+	+
7	" " " " boiled 1 min. and filtered, filtrate		—	—	—	—	—
8	" " " " " 1 " " " precipitate		—	Faint.	Slow.	+	+
9	" " boiled 1 min. + $KMnO_4$		—	Good.	+	—**	—
10	" " " 1 " " " to 65°C. and filtered		—	—	Slow.	—	—
11	" " " 1 " " " boiled again and filtered		—	—	—	—	—

* A momentary flash of light if permanganate is acid but no light in neutral or alkaline solution.

** If H_2O_2 is added to $KMnO_4$ the color disappears or if H_2O_2 is added to albumin, peptone, or gelatin + $KMnO_4$ the brown color disappears and then the blueing of guaiac will no longer take place.

TABLE I—*Continued.*

	Equal volume added to mixture of 1 part M/100 pyrogallol or 1 part 3 per cent H_2O_2 + 1 part M/100 pyrogallol; hence, concentrations in final mixture are one-half that given.	Light with pyrogallol	Light with pyrogallol + H_2O_2.	Blueing of gum guaiac	Blueing of gum guaiac + H_2O_2.	Liberation of oxygen from H_2O_2.
12	Albumin solution	—	—	—	—	—
13	" " + $KMnO_4$	—	Good.	+	—**	+
14	" " " " boiled 1 min. and filtered (no precipitate forms)	—	"	+	—**	++
15	Gelatin solution	—	—	—	—	—
16	" " + $KMnO_4$	—	Good.	—	—**	++
17	" " " " boiled 1 min. and filtered (no precipitate forms)	—	"	+	—**	++
18	Witte peptone solution	—	—	—	—	—
19	" " " + $KMnO_4$, filtered	—	Faint.	+	—**	++
20	" " " " " boiled 1 min., filtrate	—	—	—	—	—
21	" " " " " " 1 " precipitate	—	Faint.	Slow.	+	++
22	Colloidal Ag	—	Bright.	+		+
23	" Pt	—	"	+		+
24	" $Fe(OH)_3$ (dilute)	—	—	—	+	—
25	Sodium nucleoproteinate (liver)	—	—	—	+	—
26	" " (mammary gland)	—	—	—	—	—
27	" nucleate (yeast)	—	—	—	—	—
28	Squid blood (*Sepia esculenta*). Contains hemocyanin	—	Fair.			++
29	" " " " boiled	—	Good.			—
30	Lobster blood (*Palinurus japonicus*). Contains hemocyanin and tetronerythrin, a lipochrome	—	Faint.			++
31	" blood (*Palinurus japonicus*) boiled	—	Fair.			—
32	Annelid blood (*Laonome japonica*). Contains chlorocruorin	—	Good.			
33	" " " " boiled	—	—			

34	Annelid extract (*Nereis* sp.)	–	Fair.			++
35	" " (*Thelepus japonicus*)	–	Good.			++
36	" " (*Hesione reticulata*)	–	Fair.			++
37	*Chiton* sp. extract	–	"			++
38	" " " boiled	–	"			–
39	Luminous pennatulid extract (*Cavernularia haberi*)	–	–	–	+	++
40	" ostracod " (*Cypridina hilgendorfii*)	–	–			+
41	" protozoan " (*Noctiluca miliaris*)	–	–	–	–	–
42	Firefly (*Luciola viticollis*) extract, luminous organs	–	–			++
	Precipitates.					
1	Silver ferrocyanide ($Ag_2Fe(CN)_6$)	–	–	Slow.	+	Very slow.
2	Lead " ($Pb_2Fe(CN)_6$)	–	–	–	–	–
3	Ferrous " ($Fe_2Fe(CN)_6$)	–	Faint.	+		+
4	Zinc " ($Zn_2Fe(CN)_6$)	–	–	+		Very slow.
5	Copper " ($Cu_2Fe(CN)_6$)	–	–	+		+
6	Silver chromate (Ag_2CrO_4)	–	–	–	Very slow.	–
7	Lead " ($PbCrO_4$)	–	–	–	"	–
8	Barium " ($BaCrO_4$)	–	–	–	"	–
9	Chromic oxide (Cr_2O_3)	–	–	–	–	Slow.
10	" hydroxide ($Cr(OH)_3$)	–	–	–	Slow.	+
11	Manganese dioxide (MnO_2)		Good.	Slow.	"	++
12	Manganic and manganous hydroxide ($Mn(OH)_2 + Mn(OH)_3$)	–	"	"	+	+
13	Hydrated manganese hydroxide H_2MnO_3 (precipitated from $MnCl_2$ by Br + NaOH)	–	"	"	+	++

TABLE I—*Concluded.*

	Equal volume added to mixture of 1 part M/100 pyrogallol or 1 part 3 per cent H_2O_2 + 1 part M/100 pyrogallol; hence, concentrations in final mixture are one-half that given.	Light with pyrogallol.	Light with pyrogallol + H_2O_2.	Blueing of gum guaiac.	Blueing of gum guaiac + H_2O_2.	Liberation of oxygen from H_2O_2.
14	Manganic oxide (Mn_2O_3)	–	Faint.	"	+	+ +
15	Manganese metal (powder) (Mn)	–	–	"	Slow.	+
16	Metallic Ag	–	Faint.	+		+ +
17	Iron metal (Merck's reagent by hydrogen) (Fe)	–	–	–	–	Very slow.
18	Black ferrous oxide (FeO)	–	–	–	Slow.	+
19	Ferric hydroxide ($Fe(OH)_3$)	–	–	–	–	+
20	Ferrous-ferric oxide (Fe_3O_4)	–	–	–	–	+
21	Red ferric oxide (Fe_2O_3)	–	–	–	–	–
22	Copper hydroxide ($Cu(OH)_2$)	–	–	–	Slow.	+
23	Red copper oxide (Cu_2O)	–	–	Very slow.	+	+
24	Black " " (CuO)	–	–	–	+	+
25	Silver oxide (Ag_2O)	–	Faint.	+		+
26	Red mercuric oxide (HgO)	–	–	–	–	–
27	Black mercurous oxide (Hg_2O)	–	–	–	Slow.	+
28	Lead peroxide (PbO_2)	–	–	+		+ +
29	" oxide (PbO)	–	–	–	–	+
30	Red lead oxide (Pb_3O_4)	–	–	–	–	Very slow.
31	Bone black (C)	–	–	–	Very slow.	+
32	Infusorial earth (SiO_2)	–	–	–	–	–
33	Platinum black	–	Faint.	+		+

solutions which blue guaiac readily (bromine water) give no light with pyrogallol + H_2O_2, and some substances which blue guaiac + H_2O_2 only slowly (potassium chrom alum and MnO_2) give light with pyrogallol + H_2O_2. Generally the substances giving light with pyrogallol + H_2O_2 liberate visible bubbles of oxygen from H_2O_2 but this is not necessary for light production, as indicated by the plant extracts or blood which give a good light after the catalase has been destroyed. In my first paper I reported that blood containing hemocyanin gave no light but this is an error due to the use of solutions too much diluted. Note also from Table I that extracts of luminous animals give no light with pyrogallol.

Of special interest is the light produced by insoluble precipitates like MnO_2 and the colloidal metals Ag and Pt. Platinum black, with less surface, gives only a faint light. These exert a marked catalytic decomposition of H_2O_2 and they are said to be wholly unchanged at the end of the reaction. Indeed light has been observed when H_2O_2 is dropped upon finely divided MnO_2, Ag, or Pt (Dammer, 14) but with the concentrations of H_2O_2 used in my experiments light only appears if pyrogallol is also present. The Pt and Ag solutions were supplied by Dr. Goss of the Chemistry Department, whose paper on light production by colloidal solutions appears on page 271.

Potassium permanganate added to many organic substances is reduced to hydroxides, probably $Mn(OH)_3$, and with certain organic substances, especially proteins, the $Mn(OH)_3$ forms a colloidal solution. The colloidal $Mn(OH)_3$ has a marked oxidizing action. This is unquestionably the explanation of Reed's (16) supposed action of permanganate on peroxidases as indicated by the work of Bunzell and Hasselbring (15), a conclusion which I had already reached when their paper appeared. Reed supposed that the permanganate gave oxygen to the peroxidase converting it into a substance with greater oxidizing power, but the formation of colloidal $Mn(OH)_3$ explains his results satisfactorily. These colloidal Mn compounds will oxidize guaiac directly and give light with pyrogallol + H_2O_2 as will also a precipitate of $Mn(OH)_3$. My results are recorded in Table I. Although gum guaiac is oxidized without H_2O_2, no light appears with pyrogallol unless H_2O_2 is present. An excess of H_2O_2 converts the brown colloidal $Mn(OH)_3$ into colorless compounds which will then not blue guaiac.

It is a curious fact that although the ferrocyanides give a good light, the ferricyanides give none or at most a very faint light. Different samples of both ferrocyanide and ferricyanide solutions were found to vary in this regard and the variation proved to be the result of exposure of the solutions to light and air. Ferrocyanide particularly acquires the power to oxidize pyrogallol with bright light production after exposure to light and air. The phenomenon has nothing to do with phosphorescence (*i.e.*, the giving out of light rays after exposure to light) but is no doubt the result of the formation of some substance in the ferrocyanide by photochemical action. It is presumed that this substance, whatever it is, has a marked power of transferring oxygen from H_2O_2 to the pyrogallol. Photochemical changes in ferrocyanides and ferricyanides are well known and very marked and, according to Haber (22), consist in the splitting of $Fe(CN)_6''''$ to $Fe^{\cdot\cdot}$ + 6 CN'. Under the influence of light the iron separates and precipitates as $Fe(OH)_3$ or, if $(NH_4)_2S$ be added, as black FeS. However, other changes must also occur as neither iron salts nor $Fe(OH)_3$ in colloidal or precipitate form can oxidize pyrogallol with anything like the light production obtained with $K_4Fe(CN)_6$ after exposure to sunlight and air. The following tabulation gives the results of my light experiments with $K_4Fe(CN)_6$ and $K_3Fe(CN)_6$.

Solution.	Color before exposure to sunlight.	Color after exposure to sunlight 1 week.	Light production with M/100 pyrogallol + 3 per cent H_2O_2.	
			Solution exposed to sunlight.	Solution kept in the dark.
M/20 $K_4Fe(CN)_6$ in air.	Light yellow.	Deep yellow with brownish precipitate.	Bright.	Negative to very faint.
M/20 $K_4Fe(CN)_6$ in hydrogen.	"	Light yellow.	Negative.	"
M/20 $K_3Fe(CN)_6$ in air.	Yellow.	Dirty green with brown precipitate.	Fair.	"
M/20 $K_3Fe(CN)_6$ in hydrogen.	"	Dirty green with blue precipitate.	"	"

2. *Luminescence During Electrolysis.*—All attempts to obtain light from the oxidation of pyrogallol by the nascent oxygen set free at the anode during electrolysis have failed despite the fact that various oxidizers were present. Bancroft and Weiser (17) have described light at the anode during electrolysis of certain salts. An especially bright orange light was observed during the electrolysis of NaBr with the mercury anode. The color is the same as that obtained when mercury vapor burns in an atmosphere of bromine to form HgBr and the light during electrolysis is no doubt due to combination of the bromine set free by electrolysis with the mercury of the anode. A film of HgBr can be observed to form and under this film the mercury is supposed to be volatilized by the heating effect of the current.

Anode.	Electrolyte.	Light production at anode or cathode.
Platinized platinum.	Half saturated K_2SO_4 containing M/20 pyrogallol.	Negative.
" "	Half saturated K_2SO_4 containing M/200 pyrogallol.	"
" "	Turnip juice + K_2SO_4 containing M/200 pyrogallol.	"
Pt with PbO_2 surface.	Half saturated K_2SO_4 containing M/200 pyrogallol.	"
" " MnO_2 "	Half saturated K_2SO_4 containing M/200 pyrogallol.	"
Lead.	Half saturated K_2SO_4 containing M/200 pyrogallol.	"
Platinum.	$MnSO_4$ solution containing M/200 pyrogallol.	"

Reed (3) found that platinum black only blues guaiac if first exposed to nascent oxygen by making it the anode in some electrolyte. He used dilute HCl as the electrolyte, a fluid open to the objection that chlorine, whose powerful oxidizing action is so well known, is also liberated at the anode. This objection can be eliminated by using Na_2SO_4 solution as electrolyte and I can confirm Reed's result. A platinized surface made anode in a solution of Na_2SO_4 and then washed in water will blue gum guaiac directly but loses this power if made cathode in the same solution and then washed in water. Many other metallic sur-

faces will blue guaiac if made anodes but none of these give light with pyrogallol if made anodes in electrolytes containing pyrogallol. The combinations in the table on page 323 were tried. The Pt cathode which becomes markedly alkaline was enclosed in a porous cup. The current varied up to a maximum of 2 amperes.

In some experiments the Pt cathode instead of being enclosed in a porous cup was rotated rapidly but in no case did light appear.

3. Peroxides Giving Light with Pyrogallol and Oxidizers.—The peroxidase of turnip juice or $K_4Fe(CN)_6$ or any substance which gives light with pyrogallol and H_2O_2 may be spoken of collectively as oxidizers. No substance of peroxide nature other than H_2O_2 will give light of any brilliancy or permanency with these oxidizers. Certain oxidizers will give a momentary light with such peroxides as Na_2O_2. BaO_2, or perborates and persulfates but it is very faint and does not last as does the light with H_2O_2. The substances tried are listed in Table II.

TABLE II.

Peroxides Giving Light with Pyrogallol and Oxidizers.

Oxidizer. (Equal parts added to a mixture of M/100 pyrogallol and the peroxide.)	H_2O_2 3 per cent.	Benzoyl hydrogen peroxide (insoluble powder).	Ozonized turpentine (one drop).	Na_2O_2 (powder).	BaO_2 (powder).	MnO_2 (insoluble powder).	PbO_2 (insoluble powder).	K persulfate M/10.	Na perborate M/20.	K perchlorate M/10.	Quinone (insoluble crystals).
Turnip juice	+	–	–	–	–	–	–	–			–
1 per cent blood extract.	+	–	–	Faint flash.	–	–	–	–	–	–	–
M/20 $K_4Fe(CN)_6$	+	–	–	–	–	–	–	–	–	–	–
M/100 $KMnO_4$	+	–	–	–	–	–	–	Faint flash.	Fair flash.	–	–
M/10 $FeCl_3$	+								–	–	
M/100 CrO_3	+								–	–	
Na hypobromite	+	–	–	Faint flash.	Faint flash.	–	–	Fair flash.	Fair flash.	–	–
Ca hypochlorite	+	–	–	–	–	–	–	Faint.	"	–	–
MnO_2	+										
$Mn(OH)_3$ sol in peptone	+								–	–	
Colloidal Ag	+										

4. Concentration of Pyrogallol and Oxidizer and Light Production.—Pyrogallol and H_2O_2 and the oxidizer must naturally be present in a definite low concentration before light is visible. This concentration is surprisingly low for pyrogallol—M/32,000 when potato juice is used as oxidizer (Harvey, 1) and M/512,000 with $K_4Fe(CN)_6$. The latter dilution corresponds to 1 part of pyrogallol in 5,000,000 parts of water. With increasing pyrogallol concentrations the intensity of the light increases up to a limit and then decreases. In the case of vegetable peroxidases this is no doubt due in part to precipitation of the peroxidase, as a visible coagulum is formed by strong pyrogallol in potato juice. However, this explanation does not hold for $K_4Fe(CN)_6$. In the case of this oxidizer, we also find no light produced if too strong pyrogallol is used. It is also necessary to employ an optimum concentration of $K_4Fe(CN)_6$ in order to produce light. Too *great* as well as too small a concentration of $K_4Fe(CN)_6$ fails to excite luminescence. H_2O_2 must be present in a certain low concentration but there is apparently no upper limit in this case, at least to a concentration (after mixing) of 7.5 per cent. The actual concentrations necessary are given in Tables III and IV. An

TABLE III.

Concentration of Pyrogallol and Light Production with (after Mixing) 0.75 per Cent H_2O_2 and M/40 $K_4Fe(CN)_6$.

Concentration of pyrogallol (after mixing).	Character of light from mixture.
M/4	Negative.
M/40	Faint.
M/400	Good.
M/4,000	Bright.
M/8,000	"
M/16,000	"
M/32,000	Fair.
M/64,000	Faint.
M/128,000	"
M/256,000	Very faint.
M/512,000	Extremely faint.
M/1,024,000	Negative.

TABLE IV.

Concentration of $K_4Fe(CN)_6$ and Light Production with (after Mixing) 0.75 per Cent H_2O_2 and M/400 Pyrogallol.

Concentration of $K_4Fe(CN)_6$ (after mixing).	Character of light from mixture.
M/4*	Negative to very faint.
M/8	Faint.
M/16	Good.
M/32	"
M/64	Fair.
M/128	"
M/256	Faint.
M/512	Negative.

* If this solution is first exposed to sunlight a good light results.

excess (at least ten times the amount necessary) of H_2O_2 was present.

What is the explanation of this absence of light with concentrated pyrogallol or concentrated $K_4Fe(CN)_6$ solutions? We know that oxidation of the pyrogallol goes on because the mixture turns dark. One immediately thinks of absorption of light by the concentrated solutions. It is obvious that no light will be visible unless the intensity of emission is greater than the absorption. Both M pyrogallol and M/2 $K_4Fe(CN)_6$ are colored, the former a dirty light yellow, due to slight spontaneous oxidation, and the latter a clear light yellow. Neither of these solutions absorb light sufficiently to obscure the luminescence of mixtures of M/200 pyrogallol and M/20 $K_4Fe(CN)_6$ with H_2O_2, as we can very easily determine by viewing the luminescent mixture in a test-tube of 12 mm. diameter through at least a 40 mm. layer of M pyrogallol or M/2 $K_4Fe(CN)_6$. Only when the light of the luminescent mixture has markedly faded is the absorption noticeable. The oxidation products of pyrogallol, brown in color, do absorb light to a marked extent and the rapid fading of the luminescence of pyrogallol is no doubt largely due to the continued formation of these products. Absorption by brown oxidation products, which are formed only slowly, could not explain the *absence of immediate light production* on mixing M pyrogallol with H_2O_2 and M/20 $K_4Fe(CN)_6$ or M/2 $K_4Fe(CN)_6$ with M/100 pyrogallol and H_2O_2.

The explanation must lie in some other direction. Perhaps the greatest amount of light is emitted with a definite optimum velocity of oxidation of pyrogallol. The velocity of oxidation of pyrogallol should increase with increasing concentration of pyrogallol or of $K_4Fe(CN)_6$. We may suppose that with concentrated pyrogallol or concentrated $K_4Fe(CN)_6$ the velocity is above the optimum and although much heat is produced, none of the energy goes to light. If this supposition is correct we might expect to obtain light with concentrated pyrogallol or ferrocyanide by lowering the temperature, since we might in this way lower the reaction velocity to the desired optimum. Experiment has shown, however, that not lowering but on the contrary *raising* the temperature results in the greater production of light when either pyrogallol or ferrocyanide is present in *high concentration*. This

can be seen by a glance at Tables VI and VII. The phenomenon is akin to that observed during the oxidation and luminescence of phosphorus (23). White phosphorus will only luminesce in presence of oxygen but if the oxygen pressure is too great, the luminescence ceases. At 0°C. with water vapor present this "maximum luminescence pressure" of oxygen is 320 mm. Hg. and it increases 13.19 mm. Hg. for each degree rise in temperature. Thus there is a maximum and a minimum concentration of oxygen for the luminescence of phosphorus. In the case of pyrogallol we have a maximum and a minimum concentration of pyrogallol and also of the oxidizer for the luminescence of pyrogallol. There appears to be no maximum for H_2O_2. The effect of temperature is to raise the maximum and is the same both as regards phosphorus and pyrogallol.

The oxidation of phosphorus probably proceeds in several steps with the formation of intermediate oxides. If we assume that only one of these intermediate oxidations is connected with the production of light it is probable that a certain oxygen pressure and temperature will favor that reaction step at the expense of the others. This oxygen concentration and temperature will then correspond to the optimum for luminescence. A similar explanation may be applied to the oxidation of pyrogallol but this case is further complicated by the presence of an oxidizer.

Potassium ferrocyanide is not the only oxidizer which fails to give light with concentrated pyrogallol. Many others are known. These include M/100 $KMnO_4$, MnO_2 powder, M/100 CrO_3, and M/10 $FeCl_3$. On the contrary, NaClO and NaBrO give a bright flash no matter what the concentration of pyrogallol. If ozone gas is bubbled through M/100 pyrogallol (without H_2O_2) each bubble is brightly luminescent; with M/10 pyrogallol the bubbles are faintly luminescent, while no light appears if the pyrogallol is of M concentration. Pyrogallol behaves toward most of these oxidizers as toward $K_4Fe(CN)_6$ but the hypochlorites and hypobromites offer a marked exception for which no explanation is at present attempted.

5. Temperature and Light Production.—The effects of temperature have already been briefly mentioned in the preceding section. Since the velocity of most chemical reactions is doubled or tripled with each 10° rise in temperature, it is to be expected that the

TABLE V.

Temperature and Light Production. The Oxidizer Is Mixed with an Equal Amount of M/100 Pyrogallol + 3 per Cent H_2O_2.

Oxidizer.	Temperatures.				
	0–2°	20°	50°	75°	98–100°
Turnip juice	Faint.	Good.	Good.		Negative.
1 per cent blood extract	"	Fair.	"		Fair.
M/20 $K_4Fe(CN)_6$	Negative.	Good.	Bright.	Bright.	Good.
M/100 $KMnO_4$	Fair.	"	"	"	Faint flash.
M/50 $K_2Cr_2O_7$	Negative.	Fair.	Faint.	Fair.	Negative.
M/100 CrO_3	"	Good.	Bright.	Bright.	Faint.
M/10 KCr alum	"	Faint.	Faint.	Faint.	Negative.
M/10 $NH_4Fe^{\cdot\cdot\cdot}$ alum	"	"	"	"	Very faint.
MnO_2	"	Fair.	Fair.	Fair.	Negative.
NaClO	Bright flash.	Bright flash.	Bright flash.		Fair flash.

TABLE VI.

Temperature, Concentration of Pyrogallol, and Light Production. An Equal Amount of M/20 $K_4Fe(CN)_6$ *Is Mixed with Pyrogallol + 3 per Cent* H_2O_2.

Concentration of pyrogallol (after mixing).	Temperatures.						
	0–2°	10°	20°	30°	50°	75°	98–100°
M/4......	Negative.	Negative.	Negative.	Very faint.	Faint.	Fair.	Faint.
M/40.....	"	Faint.	Faint.	Faint.	Good.	Bright.	Good.
M/400....	Faint.	Fair.	Good.	Good.	"	"	Bright flash.
M/4,000 .	Bright.	Bright.	Bright.	Bright.	Bright flash.	Fair flash.	Negative.

TABLE VII.

Temperature, Concentration of Ferrocyanide, and Light Production. An Equal Amount of $K_4Fe(CN)_6$ *Is Mixed with* M/100 *Pyrogallol + 3 per Cent* H_2O_2.

Concentration of $K_4Fe(CN)_6$ exposed to light (after mixing).	Temperatures.						
	0–2°	10°	20°	30°	50°	75°	98–100°
Half saturated at 20°C........	Negative.	Faint.	Fair.	Fair.	Good.	Good.	Faint flash.
One-sixth saturated at 20°C..	Very faint.	Fair.	Good.	Good.	Bright.	Very bright.	Good "

intensity of the light will be increased and the duration of the light decreased with rise in temperature. Although no numerical data can be given, my experiments do indicate that with rise of temperature, for each oxidizer (Table V), and each concentration of oxidizer (Table VII), or each concentration of pyrogallol (Table VI) the duration of the light is shorter and its intensity is greater up to a certain definite temperature. Above this temperature the intensity as well as the duration of the light decreases.

It is perfectly plain why potato juice gives no light at high temperatures, because its oxidizer is destroyed between 80° and 85°; but why should the light be fainter or absent at 98° in the case of $KMnO_4$ or MnO_2 or any of the oxidizers of Table V which are not affected by a temperature of 98°C.? In harmony with the explanation already suggested we must suppose that it is a definite (optimum) temperature and not the highest temperature which favors that particular step in the oxidation of pyrogallol involving the production of light. At the temperature indicated in the lower right hand square of Table VI, particularly, the oxidation proceeds in such a way that no light is produced.

6. Light Production in Absence of Peroxide.—When pyrogallol is oxidized by peroxidase and hydrogen peroxide, purpurogallin, a substance of doubtful composition, is said to be formed. Its formula is probably $C_{12}H_8O_5$ (4) or $C_{11}H_8O_5$ (5). This substance can be recognized qualitatively by its dark color. As purpurogallin is fairly insoluble in cold water, Bach and Chodat (2) determined it by collection on a weighed filter, drying at 110°, and again weighing. Reed (3) made determinations by filtering, washing free of unoxidized pyrogallol, and titrating by 0.05 M $KMnO_4$.

Purpurogallin or a similar substance is also formed when pyrogallol is oxidized by many oxidizing agents; *viz.*, by $AgNO_3$, $KMnO_4$, or $NaNO_2$ in acid solution, by quinone or platinum black or $K_3Fe(CN)_6$, or by allowing a solution of pyrogallol and gum arabic to stand. Perkin (5) reports a very favorable yield by the electrolytic oxidation of pyrogallol in 15 per cent Na_2SO_4 with lead cathode and rotating platinum anode. I have observed no light during the action of these substances in moderate concentrations (*i.e.*, in concentrations comparable to those previously described as giving light in the presence of H_2O_2) although the

pyrogallol turned brown. The $AgNO_3$ was reduced to metallic Ag. The following combinations were tried.

1 cc. 2 per cent $AgNO_3$ + 1 cc. N/10 HNO_3 mixed with 2 cc. M/100 pyrogallol.

1 cc. M/10 $NaNO_2$ + 1 cc. N/10 HNO_3 mixed with 2 cc. M/100 pyrogallol.

2 " " " mixed with 2 cc. M/10 pyrogallol.

1 " " $K_3Fe(CN)_6$ + 1 cc. N/10 HNO_3 mixed with 2 cc. M/100 pyrogallol.

Gum arabic solution and M/2 pyrogallol.

The gum arabic and pyrogallol mixtures become brown slowly (during the course of 2 or 3 days) and needle crystals, sometimes curved and forming rosettes, of a golden brown substance, separate. No light was observed to be given off during this process.

The only substances investigated capable of giving light with weak pyrogallol *alone* are sodium hypochlorite and hypobromite (Table I), ozone, and acid permanganate, which gave light in the following combination.

1 cc. M/100 $KMnO_4$ + 1 cc. N to N/10 HCl or HNO_3 mixed with 2 cc. M/100 pyrogallol.

Ozonized oxygen (by silent electric discharge) bubbled through M/100 pyrogallol.

The light with acid permanganate is at most a momentary flash. Neutral or alkaline permanganate gave no light with pyrogallol alone. Fahrig (19) had observed that ozone shaken with certain samples of water would luminesce but was unable to determine the cause of it. Otto (20) found that water carefully purified of organic matter gave no light with ozone but that benzene (feeble light), alcohol, thiophene, milk, and urine did. I find that M/100 and M/10 pyrogallol, M/10 orcinol, M/10 resorcinol, M/100 esculin, fresh or boiled potato juice, and certain other fluids give light if ozone is bubbled through them. Each bubble, as it rises through the liquid is aglow. As already mentioned, if the pyrogallol is too concentrated (M solution) no light appears. Pure oxygen gives no light if bubbled through the above solutions.

Pyrogallol also turns brown if an alkaline solution is exposed to air. CO_2, acetic acid, and brown substances are said to be formed. Oxygen is absorbed, the amount depending on the amount of alkali present up to a certain concentration. Weyl

and Goth (7) found that most oxygen was absorbed when 0.25 gm. of pyrogallol was present in 10 cc. of NaOH of specific gravity 1.03. Less pyrogallol or a greater or less amount of NaOH resulted in the absorption of less oxygen. Trautz and Schorigin (8) found that a mixture of 2 cc. of 10 per cent pyrogallol + 5 cc. of 10 per cent Na_2CO_3 luminesced at the surface but I have been unable to confirm this experiment or to observe light at room temperature during the darkening of pyrogallol + alkali under any conditions. Alkali of all concentrations from solid NaOH or Na_2CO_3 to N/6,250 NaOH has been added to various concentrations of pyrogallol but light has never been observed. It is perhaps not surprising to observe no light in the more concentrated solutions, which turn black almost instantly, because of absorption, but even in weak solutions (N/10 to N/6,250 NaOH + M/100 pyrogallol) *no light has ever been observed* even though H_2O_2 also be added. Such solutions become only brown in color, the more deeply brown the greater the concentration of the alkali.

The absence of light during the absorption of oxygen in alkaline pyrogallol is possibly due to the rapidity of the oxidation. The oxidation of the pyrogallol by $K_4Fe(CN)_6$ can be made to proceed so rapidly or in such a way that no light appears (Table VI, the 98° column); on the other hand it may be that the oxidation product formed with alkali is different from that formed with H_2O_2 and an oxidizer or that one step in the oxidation series is omitted. So little is known of the chemistry of purpurogallin that we cannot decide the question at the present time. It is apparent, however, that the conditions for light production are quite definite. The oxidation must proceed in a particular way.

7. Light Production in Non-Aqueous Solvents. Alcohol.—Light production occurs in fairly strong alcohol as indicated by the mixtures in Table VIII.

The turnip juice plus alcohol will give light with pyrogallol so long as the peroxidase is not precipitated but if precipitation occurs, then only a very faint light is produced. Strong alcohol (95 per cent), however, does not prevent light production so long as an oxidizer not affected by the alcohol, such as MnO_2, is employed. Even hemoglobin powder (Eimer and Amend) gives a faint light with pyrogallol plus H_2O_2 if suspended in absolute alcohol (No. 6 of Table VIII).

TABLE VIII.

Light Production in Alcohol.
Equal Parts of Solution A and Solution B Are Mixed.

No.	Solution A. 1 cc. of 3 per cent H_2O_2 in 90 per cent alcohol, plus:		Solution B.		Light production.
	1 cc. pyrogallol.	In alcohol.	Substance.	In alcohol.	
		per cent		*per cent*	
1	M/100	Absolute.	Precipitate formed by adding 90 per cent alcohol to turnip juice.	90	Very faint.*
2	M/200	50	Same with 50 per cent alcohol.	50	Faint.*
3	M/200	50	Filtrate from above precipitation.	50	Negative.
4	M/200	40	Precipitate formed by adding 40 per cent alcohol to turnip juice.	40	Bright.
5	M/200	40	Filtrate from above precipitation.	40	"
6	M/100	Absolute.	Hemoglobin powder.	Absolute.	Faint.
7	M/200	50	" "	50	Bright.
8	M/100	Absolute.	Powdered MnO_2.	Absolute.	Fair.
9	M/100	"	" H_2MnO_3.	"	Good.
10	M/100	"	M/10 $FeCl_3$.	"	Negative.
11	M/200	50	M/20 "	50	"

* Light appears only some time after mixing.

Acetone.—Hemoglobin or MnO_2 powder both give light with 50 per cent acetone containing M/200 pyrogallol and 1.5 per cent H_2O_2. The light from the hemoglobin is very bright. If benzoyl peroxide replaces the H_2O_2 no light appears.

Ether, Chloroform, and Benzene.—Pyrogallol is slightly soluble in benzene, fairly soluble in chloroform, and easily soluble in ether. If pyrogallol (M/100 or less) in ether, chloroform, or benzene be shaken with an equal volume of 3 per cent H_2O_2 in water, the ether, chloroform, and benzene layer then removed, and added to hemoglobin or MnO_2 powder, light only appears with ether and hemoglobin, and it is very faint. Benzoyl peroxide in place of H_2O_2, although soluble in all three solvents, will give no light in any case.

8. *Effect of Acid and Alkali on Light Production.*—The influence of acid and alkali on light production can best be studied by using as an oxidizer some inert substance such as MnO_2 not changed by acid or alkali in dilute concentration. Table IX gives the results. The MnO_2 powder was added to the HCl and NaOH in the concentrations indicated in the table and mixed with an equal volume of 1 cc. of 3 per cent H_2O_2 + 1 cc. M/100 pyrogallol. The concentration of acid and alkali in the final mixture is therefore one-half of that designated in the table. It will be observed that a small concentration of acid (N/160) inhibits the light, whereas a definite concentration of alkali (N/40) increases the light to an optimum.

TABLE IX.

Effect of HCl and NaOH on Light Production by MnO_2.

Concentration of HCl.	Light production.	Concentration of NaOH.	Light production.
N/10	Negative.	N/10	Fair.
N/20	"	N/20	Bright.
N/40	"	N/40	Fair.
N/80	Very faint.	N/80	Faint.
N/160	Faint.	N/160	"
Water.	"	Water.	"

SUMMARY.

1. The literature on chemiluminescence of pyrogallol is reviewed and a classification of all known types of chemiluminescent reactions given. A light-producing system consisting of an oxidizer, a peroxide, and an oxidizable substance (pyrogallol) is considered in detail.

2. In addition to substances already recorded, chromates, bichromates, hypochlorites, hypobromites, hypoiodites, chromium and iron salts, colloidal silver, platinum, and oxides of manganese, bloods containing hemocyanin or chlorocruorin, and precipitates of $Fe_2Fe(CN)_6$, $Mn(OH)_2$ + $Mn(OH)_3$, MnO_2, Mn_2O_3, Ag_2O, and metallic silver and platinum black all give light with pyrogallol and H_2O_2. Many other substances recorded gave no light.

3. Ferrocyanide solutions only give a bright light with pyrogallol + H_2O_2 if first exposed to sunlight and air; ferricyanides give

a faint light only, if exposed to sunlight in presence or absence of air.

4. Perborates, persulfates, Na_2O_2, and BaO_2 can take the place of H_2O_2 with some oxidizers but not with all.

5. No light has ever been observed during the liberation of nascent electrolytic oxygen at anodes of platinum black, manganese dioxide, or lead peroxide, in electrolytes containing pyrogallol.

6. Pyrogallol gives light in absence of peroxide, only with sodium hypochlorite and hypobromite, acid (but not neutral or alkaline) permanganate, and ozone. No light has been observed in presence of alkali (with or without H_2O_2) although rapid oxidation takes place.

7. There is a maximum, minimum, and optimum concentration of pyrogallol and also of potassium ferrocyanide for light production. Above the maximum and below the minimum no light appears. No maximum was found for H_2O_2, up to a concentration of 7.5 per cent.

8. Increase of temperature increases the brightness of the light for each concentration of pyrogallol and of potassium ferrocyanide up to an optimum; then a decrease occurs. The greater the concentration of pyrogallol or of ferrocyanide, the higher the temperature necessary to give light. Increase of temperature decreases the duration of the light.

9. Pyrogallol can be oxidized with light production in fairly strong alcohol, acetone, and ether.

10. Acid prevents and alkali favors the luminescent oxidation of pyrogallol by $MnO_2 + H_2O_2$.

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THE STRUCTURE OF THE PURINE MONONUCLEOTIDES.

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A careful examination of the literature on the structure of plant nucleic acid will show that excellent evidence has been furnished for the following conclusions.

1. Nucleic acid is a tetranucleotide; that is to say, it is composed of the groups of four mononucleotides.

2. The four mononucleotide groups of yeast nucleic acid are joined to one another through their carbohydrate groups, giving rise to a polysaccharide structure. The order of the mononucleotide groups in nucleic acid has been determined.

These two deductions are based principally upon the preparation and properties of various simpler nucleotides from yeast nucleic acid,[1] and upon a comparative study of the rate at which phosphoric acid is liberated from them by mild acid hydrolysis.[2]

3. Each mononucleotide is composed of three groups, because by complete acid hydrolysis each produces three substances, ribose, phosphoric acid, and a nitrogenous compound. This has been found in the case of one of the four mononucleotides (guanine mononucleotide) by direct experiment with the substance itself but its extension to the other three mononucleotides, although indirect, is practically conclusive.

[1] Guanine-cytosine dinucleotide, Jones, W., and Richards, A. E., *J. Biol. Chem.*, 1915, xx, 25.

Adenine-uracil dinucleotide, Jones, W., and Read, B. E., *ibid.*, 1917, xxix, 111.

Uracil-cytosine dinucleotide, Jones and Read, *ibid.*, 1917, xxxi, 39.

Guanine mononucleotide, Read, *ibid.*, 1917, xxxi, 47.

[2] Jones and Read, *J. Biol. Chem.*, 1917, xxix, 123.

4. The preparation of four nucleosides from plant nucleic acid proves that in each of the four mononucleotides the nitrogenous group is combined to a ribose group.[3]

These statements would deal completely with the *gross* structure of nucleic acid as represented in the formula below, if the positions of the phosphoric acid groups in the four mononucleotides were definitely known.

$$
\begin{array}{lll}
HO\diagdown & & \\
\quad O{=}P - O\,.\,C_5H_7O_2 & . & C_5H_4N_5 \\
HO & \quad\;| & \\
 & \quad\;O & \\
HO & \quad\;| & \\
\quad O{=}P - O\,.\,C_5H_6O & . & C_4H_3N_2O_2 \\
HO\diagup & \quad\;| & \\
 & \quad\;O & \\
HO\diagdown & \quad\;| & \\
\quad O{=}P - O\,.\,C_5H_6O & . & C_4H_4N_3O \\
HO\diagup & \quad\;| & \\
 & \quad\;O & \\
HO\diagdown & \quad\;| & \\
\quad O{=}P - O\,.\,C_5H_7O_2 & . & C_5H_4N_5O \\
HO\diagup & &
\end{array}
$$

Yeast nucleic acid.

We propose to show the position of the phosphoric acid group in each of the purine mononucleotides.

The three possible arrangements of the three groups in guanine mononucleotide are indicated in the following formulas.

$$
\text{I.}\qquad HO - \overset{\displaystyle O}{\overset{\|}{\underset{\displaystyle OH}{\underset{|}{P}}}} - O\,.\,C_5H_8O_3\,.\,C_5H_4N_5O
$$

$$
\text{II.}\qquad C_5H_9O_4\,.\,O - \overset{\displaystyle O}{\overset{\|}{\underset{\displaystyle OH}{\underset{|}{P}}}}\,.\,C_5H_4N_5O
$$

[3] Guanosine, Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, 1909, xlii, 2474; adenosine, 2703; cytidine, *ibid.*, 1910, xliii, 3150.

Uridine, Levene, P. A., and La Forge, F. B., *Ber. chem. Ges.*, 1912, xlv, 608. Read, B. E., and Tottingham, W. E., *J. Biol. Chem.*, 1917, xxxi, 295.

$$\text{III.} \qquad C_5H_9O_4 \, . \, C_5H_3N_5O \, . \, \overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle OH}{|}}{P}} - OH$$

Guanine mononucleotide forms guanosine, so that its guanine and ribose groups must be directly combined. Moreover, the nucleotide is a dibasic acid that forms a crystalline dibrucine salt. Formula II is therefore excluded.

A crucial experiment described below decides between Formulas I and III. Guanine mononucleotide liberates guanine with much greater rapidity than it liberates phosphoric acid. Formula III is therefore excluded and Formula I alone remains.

Similar reasoning would show that adenine mononucleotide has an analogous structure. But adenine mononucleotide has never been prepared. We have found, however, that adenine-uracil dinucleotide, which can be easily obtained, liberates its adenine far more rapidly than it liberates its phosphoric acid. As the two phosphoric acid groups of the dinucleotide are not directly joined to one another the results obtained may be safely applied to hypothetical adenine mononucleotide.

Guanine Mononucleotide.

Pure guanine mononucleotide was prepared from yeast nucleic acid and allowed to dry in a desiccator with sulfuric acid. The product still contained about 7 per cent of moisture but its state of dryness and even its condition of purity are not essential to the experiments described, as we shall deal with ratios.

Six portions from the same specimen of the nucleotide were weighed into small flasks provided with condensing tubes and each portion was treated with twenty parts of 5 per cent sulfuric acid. The flasks were then submerged in a boiling water bath and heated for various periods from 5 minutes to 3 hours. Each hydrolyzed product was made alkaline with ammonia while still hot, for the precipitation of free guanine, and after standing over night the guanine was filtered off, allowed to dry, and weighed. The filtrates from guanine were heated to the boiling point and treated with magnesia mixture for the precipitation of free phosphoric acid. After standing over night the crystalline

precipitates of magnesium ammonium phosphate were filtered off, allowed to dry, and weighed.

In Table I the quantities of the constituents determined are all calculated for 1 gm. of mononucleotide and the maximum amount obtained in any experiment is assumed to be 100 per cent. From this amount the corresponding percentages for the other experiments are reckoned.

The difference in the rates at which guanine and phosphoric acid are liberated from the nucleotide is so great that it can be attributed neither to imperfections of method nor to analytical error.

TABLE I.

Nucleotide used.	Time of hydrolysis.	Magnesium ammonium phosphate.			Guanine.		
		Obtained.	Per gm. of nucleotide.	Per cent of total.	Obtained.	Per gm. of nucleotide.	Per cent of total.
0.4234	5 min.	0.0499	0.1178	18.8	0.0835	0.1972	50.0
0.5030	15 "	0.1348	0.2680	42.8	0.1747	0.3473	88.1
0.5000	30 "	0.2004	0.4008	64.0	0.1901	0.3802	96.3
0.3487	1 hr.	0.1881	0.5395	86.2	0.1396	0.4002	101.5
0.3360	2 "	0.2017	0.6000	95.8	0.1310	0.3899	98.9
0.3448	3 "	0.2158	0.6259	100.0	0.1360	0.3944	100.0

The results given in Table I are represented diagrammatically in Fig. 1. Its method of construction is so obvious as to require no explanation.

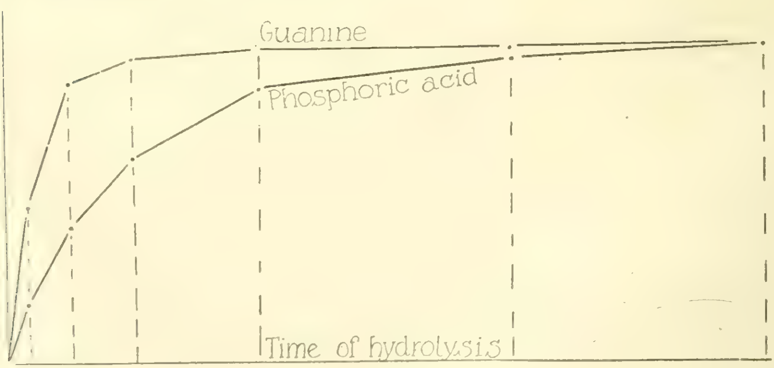

FIG. 1.

Adenine-Uracil Dinucleotide.

Portions of the dinucleotide were heated with dilute sulfuric acid for various periods of time. As guanine was not to be taken into consideration the fluids were made alkaline with ammonia and the free phosphoric acid was determined by precipitating directly with magnesia mixture as described above.

In a second series of experiments with the same specimen of dinucleotide, the hydrolyzed product was made alkaline with ammonia and the adenine was precipitated with an ammoniacal solution of silver nitrate. The gelatinous precipitates of silver-adenine were washed until the wash water contained no trace of either free or combined ammonia, when the filter papers with their precipitates were placed in Kjeldahl flasks and the nitrogen was determined. For each experiment 10 cc. of sulfuric acid, 5 gm. of potassium sulfate, and a few drops of aqueous copper sulfate were used.

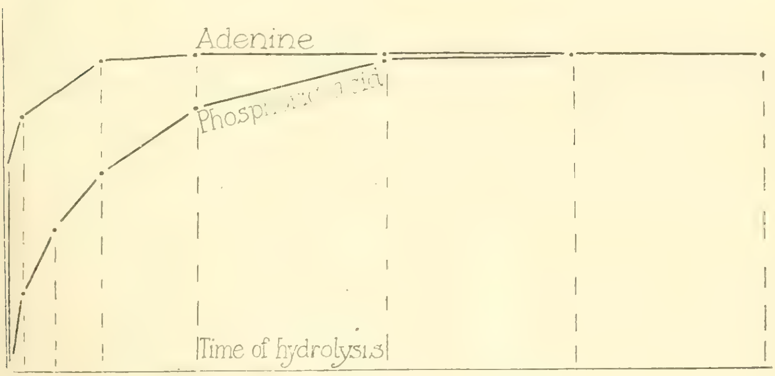

Fig. 2.

The results are given in Tables II and III and are represented diagrammatically in Fig. 2. The methods of tabulation and construction are the same as those used above for guanine mononucleotide with one exception. In the case of the dinucleotide a correction has been made for the small amount of phosphoric acid which is liberated from the pyrimidine nucleotide group. Repeated experiment has shown that this corresponds to 10 mg. of magnesium ammonium phosphate per gm.-hour.[4]

[4] Jones, W., *J. Biol. Chem.*, 1916, xxiv, p. iii. Jones and Read, *ibid.*, 1917, xxix, 111, 123.

TABLE II.

Nucleotide used.	Time of hydrolysis.	Magnesium ammonium phosphate.				
		Obtained.	Per gm. of nucleotide.	Pyrimidine correction.	From purine nucleotide.	Per cent of total.
0.7665	5 min.	0.0726	0.095	0.001	0.094	25.3
0.9712	15 "	0.1638	0.169	0.002	0.167	44.9
0.9754	30 "	0.2330	0.239	0.005	0.234	62.9
0.8832	1 hr.	0.2815	0.319	0.010	0.309	83.1
0.8258	2 hrs.	0.3192	0.387	0.020	0.367	98.7
0.9500	3 "	0.3816	0.402	0.030	0.372	100.0
0.9976	4 "	0.4067	0.408	0.040	0.368	98.9

TABLE III.

Nucleotide used.	Time of hydrolysis.	Adenine nitrogen.		
		Obtained.	Per gm. of nucleotide.	Per cent of total.
0.5245	1 min.	0.0313	0.0597	69.1
0.5592	5 "	0.0393	0.0703	81.4
0.6016	30 "	0.0513	0.0853	98.8
0.5009	1 hr.	0.0436	0.0870	100.8
0.5756	2 "	0.0496	0.0861	99.7
0.5338	3 "	0.0461	0.0864	100.0

THE SIMILARITY OF THE ACTION OF SALTS UPON THE SWELLING OF ANIMAL MEMBRANES AND OF POWDERED COLLOIDS.

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I.

1. When dry animal membranes, like pig's bladder freed from fat by prolonged treatment with hot ether (a week or 10 days in a Soxhlet apparatus), are put into water, they will swell; they will also swell and even slightly more when put into a salt solution; but when the membrane is put first for about ½ hour into M/8 solution of NaCl and subsequently into distilled water, the swelling will be twice or three times as great as in either of the other two cases. A further investigation of this problem suggested a possible connection with the structure of the membrane. An investigation of this phenomenon seemed desirable in view of the fact that Flusin[1] has tried to connect the phenomena of osmosis with the imbibition of the separating membrane.

A series of pieces of dry pig's bladder, weighing about 0.4 gm. each, were put into 50 cc. of M/8 NaCl, and left there for half an hour. One was put as a control into distilled water. After a half an hour each piece was freed from adhering solution by putting it between two sheets of filter paper and pressing gently; it was then weighed. The pieces which had been in M/8 NaCl were then put into NaCl solutions of different concentrations, namely, M/8, M/32, M/128, M/512, M/1024, M/4096, M/8192 NaCl, and into H_2O, and the control in H_2O was again put into H_2O. After different intervals the membranes were taken out of the solutions, freed from liquid adhering to their surface, and weighed. Table I gives the results of one such experiment.

[1] Flusin, *Ann. Chim. et Phys.*, 1908, series 8, xiii, 480.

TABLE I.

After	Swelling of dried pig's bladder in per cent of original weight of piece of bladder.								
	In NaCl								Control H_2O
	M/8	M/8	M/8	M/8	M/8	M/8	M/8	M/8	
hrs.									
½	187	186	190	191	184	185	173	183	149
	Then transferred into NaCl								
	M/8	M/32	M/128	M/512	M/1024	M/4096	M/8192	H_2O	H_2O
1½	204	219	289	329	310	328	301	354	165
16½	219	234	363	409	408	455	438	500	187
24	240	242	396	464	451	510	542	562	208

These figures show the following facts: A dried membrane kept permanently in H_2O increases in weight 208 per cent in 24 hours, and when kept permanently in M/8 NaCl it increases in 24 hours slightly more, namely, 240 per cent. This was to be expected on the basis of the old experiments of Hofmeister. What the writer did not expect is the fact that if a piece of membrane is put first for 30 minutes into M/8 NaCl and subsequently for 24 hours into a weaker NaCl solution or distilled water, it swells the more the lower the concentration. Thus a membrane kept for 30 minutes in M/8 NaCl increased its weight when put subsequently into H_2O 562 per cent within 24 hours. The fact that a membrane kept permanently in M/8 NaCl swells so much less (240 per cent) must then be due to the fact that presence of the NaCl solution counteracts the swelling. We must, therefore, discriminate between two effects of the salt solution upon the swelling of a membrane, namely, *first*, a chemical reaction between the membrane and the salt, which would cause in itself a considerable swelling if it were not inhibited by the *second* effect of the solution which counteracts this tendency to swell, and the more so the higher the concentration of the salt solution. We can eliminate this second factor by allowing the membrane to react with the salt first and then transferring the membrane to H_2O after having washed off the adherent salt solution. In a recent series of papers the writer[2] has pointed out the different behavior of

[2] Loeb, J., *J. Biol. Chem.*, 1916, xxvii, 339, 353, 363; 1916–17, xxviii, 175.

the membrane of the eggs of *Fundulus* when taken directly from a salt solution or when taken from distilled water or a solution of a non-electrolyte.

2. When an attempt was made to repeat the experiment just described on pig's bladder with solid blocks of gelatine, either dry or containing varying quantities of water, they failed completely. Gelatine shows none of the peculiarities mentioned in Table I.

A 40 per cent gelatine solution was prepared and after it had set, small pieces were cut out of the jelly and exposed to air for 21 hours during which time they lost about 50 per cent in weight. Then the same experiment as represented in Table I was repeated. Table II gives the result.

TABLE II.

After	Increase in weight of blocks of gelatine in per cent of original weight.								
	In NaCl								Control H_2O
	M/8	M/8	M/8	M/8	M/8	M/8	M/8	M/8	
hrs.									
$\frac{1}{2}$	23	24	27	27	25	25	30	24	19.5
	Then transferred into NaCl								
	M/8	M/32	M/128	M/512	M/1024	M/4096	M/8192	H_2O	H_2O
$1\frac{1}{2}$	51	52	56	54	51	52	58	50	44
19	198	193	196	190	176	176	158	170	162
24	246	236	239	231	214	216	208	214	188

The swelling in M/8 NaCl was slightly greater than in H_2O, but the gelatine first treated with M/8 NaCl and then put into H_2O for 24 hours did not swell more than the piece kept permanently in M/8 NaCl.

Thin sheets of gelatine behaved like blocks of the same material.

It was thought that the salt had perhaps not entered sufficiently into the gelatine. To avoid this possibility the gelatine was dissolved in M/8 NaCl instead of in H_2O and after setting the pieces were cut out of the gelatine. When put into NaCl solutions varying from M/8 to M/8192 or into H_2O the result was identical with the one expressed in Table II. Nor were the results different when the gelatine used was completely dried beforehand.

3. It was found that finely powdered gelatine behaved exactly like pig's bladder and behaved differently from solid blocks of gelatine. This was true when the powdered gelatine used was the same as that used for making the solid blocks mentioned in Table II.

Commercial Cooper's powdered gelatine was put through a No. 60 sieve and again through a No. 80 sieve. The grains going through the former but not through the latter sieve served for the experiment. 2 gm. of such gelatine were put into a cylindrical funnel, the bottom of which was covered with a round piece of filter paper. The upper surface of the powdered gelatine in the funnel was also covered with a round piece of filter paper in order to make it possible to pour water or salt solution on the gelatine without stirring up the particles too much. When 25 cc. of distilled water are poured on the gelatine, part of the water runs quickly through but part of the water is retained and the mass of gelatine swells. When the process is repeated, only a slight further swelling takes place, and after this no further swelling takes place no matter how much water filters through the gelatine. When instead of letting water run through the gelatine we let 25 cc. of M/8 NaCl run through, and repeat the process of filtering M/8 NaCl through the gelatine, the latter swells also and even a trifle more than in the H_2O experiment, but the maximal swelling is also soon obtained. If, however, we allow first 25 cc. of M/8 NaCl to run through a mass of powdered gelatine and follow this with consecutive washings by distilled water, the mass will swell considerably more with each consecutive washing than in either of the other cases.

The following experiment is the analogue of the one presented in Table I. Eight cylindrical funnels, each containing 2 gm. of powdered Cooper's gelatine (size of grain between sieves No. 60 and No. 80) were prepared in the way described above, and 25 cc. of M/8 NaCl were percolated through each funnel. The mass of gelatine increased in each cylindrical funnel about 18 to 20 mm. in height. After this 100 cc. of a different solution were sent through each of the eight funnels and the additional swelling was ascertained in each mass at the end of the experiment. These solutions were: M/8, M/32, M/128, M/512, M/1024, M/4096, M/8192 NaCl, and H_2O. The results are given in Table III.

TABLE III.

After	Swelling of powdered gelatine in mm. height of a cylinder containing the powder.								
	In NaCl								Control H_2O
	M/8	M/8	M/8	M/8	M/8	M/8	M/8	M/8	
½ hr..........	19.5	20	20	19	17	18	18.5	20	15
	Then 100 cc. of the following solutions were allowed to run through.								
	NaCl							H_2O	H_2O
	M/8	M/32	M/128	M/512	M/1024	M/4096	M/8192		
Additional swelling....	8(?)	5	5	12	21	31	40	64	14

The increase in swelling is expressed in terms of the height of the cylindrical mass of gelatine.

It is obvious that when powdered gelatine is treated first with M/8 NaCl and subsequently with H_2O it retains much more water than when it is perfused permanently with H_2O or permanently with M/8 NaCl. This can also be explained on the assumption that the phenomenon is due to two different effects, first the reaction between salt and gelatine which increases the swelling, and second the inhibition of the swelling if the gelatine is washed in the salt solution. This inhibition reminds us of the inhibition of acid swelling of gelatine by the presence of salts, though the mechanism may be different in the two cases.

From these experiments it is obvious that pig's bladder and powdered gelatine have a peculiarity in common which we were not able to discover in solid blocks of gelatine, namely, to swell considerably more when a short treatment with M/8 NaCl is followed by a treatment with H_2O, than when the mass is treated exclusively with H_2O or exclusively with salt. The property which powdered gelatine and pig's bladder have in common is that they consist of very small discrete particles, grains in the one and fibers in the other; while the gelatine in a block must be considered as a homogeneous mass or as one enormous particle. It should also be stated that this peculiar behavior of powdered gelatine is probably found in many powdered colloids; thus powdered ovomucoid kindly given to us by Dr. López-Suárez behaved exactly like powdered gelatine.

To make the demonstration complete we should add that the after effect of a previous salt treatment just described is found also if other concentrations of NaCl than M/8 are used, namely, M/4, M/2, M/1, etc., but that the after effect ceases when the NaCl concentration is too low, namely, below M/64 or M/128 NaCl.

It should also be said that if we leave powdered colloids or pig's bladder permanently in a salt solution nothing comparable to this after effect is noticed even if the NaCl solution is very weak. In such cases the maximum swelling is soon reached. Table IV gives the results of such an experiment with powdered gelatine, which covers also the case for pig's bladder. We have

TABLE IV.

	Swelling of powdered gelatine in mm. height of a cylinder.											
	NaCl											H_2O
	2 M	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	
After first 25 cc. of solution.	23	22	21	24	22	20	21	21	20	20	21	19
Additional swelling after further 25 cc. of solution.	11	8	6	4	4	2	2.5	2	2	2	2	2
Additional swelling after third 25 cc. of solution....	0	0	0	0	0	0	0	0	0	0	0	0

mentioned the fact that if only M/8 NaCl solutions are allowed to percolate through powdered gelatine the maximum swelling is soon reached and no further swelling takes place. This is true for all concentrations of NaCl tried.

We only notice a slightly increased effect with the increase of the concentration.

II.

The Effect of Different Ions.

1. The excessive swelling, observed in pig's bladder, in powdered gelatine, or in ovomucoid when a short treatment with NaCl solution of not too low a concentration is followed by a treatment with distilled water, is not confined to NaCl, but is produced by

many if not all neutral salts with a univalent cation; while the neutral salts with bivalent cations have no such effect. Pieces of pig's bladder were put for 30 minutes into M/8 LiCl, NaCl, KCl, $MgCl_2$, $CaCl_2$, and $SrCl_2$. Table V gives the result.

TABLE V.

After	Increase in weight of pig's bladder in per cent of original weight in M/8 solutions of:						
	LiCl	NaCl	KCl	$MgCl_2$	$CaCl_2$	$SrCl_2$	Control H_2O
hrs.							
½	181	193	186	225	164	162	139
	They were then transferred to distilled water.						
½	306	335	325	169	138	149	145
4	592	547	454	189	138	161	151
22½	662	598	434	195	134	165	145

Leaving aside minor differences, it is obvious that the previous treatment of the membrane with Li, Na, and K causes a considerable increase in swelling, while this increase is lacking in the case of Mg, Ca, and Sr. The salts of the alkali earth simply prevent the subsequent increase in the swelling caused by NaCl or LiCl. Mg is less active than Ca or Sr, which was to be expected. *It cannot be said that $CaCl_2$ or $SrCl_2$ affect the swelling in the opposite way from that caused by NaCl, since the effect of $CaCl_2$ does not differ much from that of distilled water.*

All salts of Na cause the after effect though the quantity of swelling varies with different anions (Table VI).

TABLE VI.

After	Swelling of pig's bladder in per cent of original weight in solutions of different sodium salts.							
	M/8 NaCl	M/8 $NaNO_3$	M/8 $NaCH_3COO$	M/8 Na_2SO_4	M/16 Na_2SO_4	M/8 Na_2 tartrate	M/16 Na_2 tartrate	Control H_2O
hrs.								
½	188	209	157	195	222	172	201	167
	They were then transferred to distilled water.							
½	325	312	290	345	464	336	418	189
5	638	464	505	644	846	644	872	221
22½	758	840	490	659	832	1,071	1,400	223

The experiment shows clearly that a short treatment of the membrane with any sodium salt causes a considerable further swelling in H_2O after the free salt solution is leached out. One fact, however, stands out, namely, that the striking difference between the action of univalent and bivalent cations is in no way repeated among the anions. This is in harmony with the writer's first extensive experiments on antagonistic salt action on *Fundulus* in which he showed that the toxic action of high concentrations of salts with univalent cations could be inhibited by small quantities of salts with a bivalent cation, while no such valency effect could be found for the anions.[3]

2. The same difference in the effect of the salts with univalent and bivalent cations upon the subsequent swelling of pig's bladder in distilled water can be found in powdered gelatine. 2 gm. of powdered gelatine (of grain size 60–80) were put into each of a series of cylindrical funnels, and 25 cc. of M/8 LiCl, NaCl, KCl, $MgCl_2$, $CaCl_2$, $SrCl_2$, and $BaCl_2$ were kept in contact with the gelatine for half an hour and then allowed to run off. Four times in succession 25 cc. of distilled water were then allowed to run through each cylinder. Table VII gives the swelling in mm. height of the cylinder.

TABLE VII.

	Swelling in mm. height of cylinder of powdered gelatine under the influence of different chlorides.							
	M/8 LiCl	M/8 NaCl	M/8 KCl	M/8 $MgCl_2$	M/8 $CaCl_2$	M/8 $SrCl_2$	M/8 $BaCl_2$	Control H_2O
Swelling under influence of 25 cc. salt solution......	23	25	24	24	24	24.5	24	24
Additional swelling under influence of 100 cc. H_2O (four washings).........	15	33	25	4.5	4	3.5	2.5	5.5

The amount of swelling in the different salt solutions was too small to be discovered. In the after effect, however, the striking difference between the salts with univalent and bivalent cations

[3] Loeb, *Arch. ges. Physiol.*, 1901, lxxxviii, 68; *Am. J. Physiol.*, 1901–02, vi, 411.

(which was also noticeable in the case of the swelling of pig's membrane) shows itself. There is practically no difference in the after effect of $CaCl_2$ upon the swelling of the membrane in distilled water and the swelling caused in distilled water without any previous salt treatment.

3. No difference of this kind in the after effect of a treatment with NaCl and $CaCl_2$ could be discovered in solid blocks or sheets of gelatine.

III.

Antagonistic Salt Action.

1. In 1901[3] the writer showed that the injurious effects which a salt with univalent cation has upon the eggs of *Fundulus*, as soon as the concentration of the salt exceeds a certain concentration, can be inhibited by the addition of a very small quantity of a salt with a bivalent cation; while the addition of a salt with a bivalent anion had no such effect. The writer drew from these facts the conclusion that the antagonistic action of the bivalent cation in this case must be due to an action of the salts upon the state of colloids, but for a long time it was found difficult to imitate such an antagonistic salt action directly on colloids. Recently analogies have been shown to exist by Schryver[4] on gels of cholate solutions, by Clowes for soaps,[5] by Lenk[6] for the swelling of gelatine, and by Fenn[7] for the precipitation of dissolved gelatine by alcohol in the presence of different salts.

A very striking antagonistic salt action can be demonstrated in the case of the after effect of NaCl upon the subsequent swelling of pig's bladder and powdered colloids in distilled water. This swelling is inhibited if a comparatively small quantity of

[4] Schryver, S. B., *Proc. Roy. Soc., Series B*, 1914, lxxxvii, 366; 1916, lxxxix, 176. Schryver, S. B., and Hewlett, N., *ibid.*, 1916, lxxxix, 361.

[5] Clowes, G. H. A., *J. Phys. Chem.*, 1916, xx, 407; *Science*, 1916, xliii, 750.

[6] Lenk, E., *Biochem. Z.*, 1916, lxiii, 15, 58.

[7] Fenn, W. O., *Proc. Nat. Acad. Sc.*, 1916, ii, 534, 539. In one of the examples mentioned by Fenn, *e.g.*, the precipitation of gelatine in mixtures of solutions of Na_3 citrate and $CaCl_2$ by alcohol, the precipitation depends to a large extent upon the formation of a supersaturated solution of calcium citrate which is precipitated by alcohol even if no gelatine is present.

$CaCl_2$ (or any other salt with bivalent cations) is added to the NaCl.

The experiment consisted in the following. Pieces of dry pig's bladder were put for 30 minutes into the following solutions:

50 cc. M/8 NaCl					
50 " M/4 "	+ 1 cc.	M/8 $CaCl_2$	+ 49 cc.	H_2O
50 " M/4 "	+ 2 "	M/8 "	+ 48 "	"
50 " M/4 "	+ 4 "	M/8 "	+ 46 "	"
50 " M/4 "	+ 8 "	M/8 "	+ 42 "	"
50 " M/4 "	+ 16 "	M/8 "	+ 34 "	"
50 " M/4 "	+ 32 "	M/8 "	+ 18 "	"

As the reader will see, each of these solutions was M/8 in regard to NaCl but contained increasing concentrations of $CaCl_2$. After the membranes had been in the solutions for 30 minutes they were transferred to distilled water. It will be seen that the membrane previously treated with pure NaCl swelled considerably while the ones treated previously with NaCl + $CaCl_2$ swelled the less the more $CaCl_2$ was added.

TABLE VIII.

After	Antagonism between NaCl and $CaCl_2$ on the subsequent swelling of pig's bladder when put into distilled water. Increase in weight in per cent of original weight.							
	Cc. M/8 $CaCl_2$ in 100 cc. M/8 NaCl							Control H_2O
	0	1	2	4	8	16	32	
hrs.								
$\frac{1}{2}$	189	173	182	185	157	159	164	143
	The membranes were then all transferred to distilled water.							
3	336	305	339	280	155	155	160	
21	345	345	(430?)	269	137	154	167	145

It is obvious that the addition of 4 cc. of M/8 $CaCl_2$ to 100 cc. of M/8 NaCl already inhibits markedly the subsequent swelling of the membrane in distilled water and that the addition of 8 cc. of M/8 $CaCl_2$ to 100 cc. of M/8 NaCl inhibits this swelling almost completely.

If the reader will look over the first row of figures representing the swelling of the membranes while *in* the salt solutions the an-

tagonistic salt action will be seen to be extremely small if it exists at all.

It should also be pointed out once more that the antagonistic action between NaCl and $CaCl_2$ is not the algebraic mean between a swelling effect of NaCl and a dehydrating effect of $CaCl_2$. Table V has already contradicted such an assumption. In membranes not treated previously with any salt the swelling in H_2O amounted in Table VIII to 145 per cent, while in membranes which had been treated for 30 minutes with 100 cc. of M/8 NaCl + 32 cc. of M/8 $CaCl_2$ and were then transferred to H_2O it amounted to 167 per cent. The addition of $CaCl_2$ served only to prevent the enormous increase in weight which a previous treatment with M/8 NaCl alone induces, namely, in this experiment 345 per cent, but it cannot be said that $CaCl_2$ and NaCl affect the membrane in an opposite sense.

2. The antagonism experiment just described is successful also with powdered gelatine. 2 gm. of powdered gelatine (size of grain 60–80) were put into a series of funnels (as described in the beginning of this paper) and first 25 cc. of M/8 NaCl with different quantities of $CaCl_2$ (as described in the experiment with pig's bladder) were allowed to run through the gelatine.

Subsequently 75 cc. of distilled water were allowed to run through each cylinder. The result was striking, inasmuch as *in* the salt solution the swelling was approximately the same in pure NaCl and in NaCl with $CaCl_2$; but in the subsequent treatment with H_2O the swelling was enormous in the powdered gelatine treated previously with pure M/8 NaCl, while this after effect was prevented when 8 cc. or more of $CaCl_2$ were added to 100 cc. M/8 NaCl (Table IX).

Mg and Sr act similarly to $CaCl_2$.

The same antagonistic effect was observed with powdered ovomucoid.

3. The writer has not been able to find such an antagonistic action in the after effect of a previous treatment of solid blocks of gelatine with mixtures of NaCl and $CaCl_2$. The experiment in Table X was performed with solid pieces of dry gelatine.

While in the salt solution the swelling was greater in both M/8 NaCl and M/8 $CaCl_2$ than in pure distilled water, in the subsequent treatment with distilled water no after effect of the na-

ture of that found in powdered gelatine or in pig's bladder could be discovered.

This difference in behavior between powdered gelatine and gelatine in a solid block cannot be ascribed to a difference in the chemical nature of the gelatine used in both cases since the results

TABLE IX.

	Swelling of powdered gelatine in mm. height of a cylinder.							
	Cc. M/8 $CaCl_2$ in 100 cc. M/8 NaCl							Control H_2O
	0	1	2	4	8	16	32	
After 25 cc. salt solution had been added..........................	22	20	23	22	23	21	21	19
	Then 75 cc. distilled water were allowed to filter through.							
Additional swelling after the 75 cc. distilled water had percolated..........................	30	37	29	20	3.5	3.5	5	3

TABLE X.

After	Increase in weight of solid blocks of dry gelatine in per cent of the original weight of the solution.							
	Cc. M/8 $CaCl_2$ in 100 cc. M/8 NaCl						Controls.	
	0	1	2	4	8	16	100 cc. M/8 $CaCl_2$	H_2O
hrs. ½	101	108	99	106	113	99	110	96
	The pieces of gelatine were then transferred to distilled water.							
3	246	265	243	264	265	241	267	257
23	483	550	469	514	524	500	527	538

were the same when the blocks of gelatine were made by dissolving Cooper's powdered gelatine in warm water and allowing the solution to set. The results were also the same when the solid blocks of gelatine were only half dry at the beginning of the experiment, or when they were exposed to the salt solution longer or less than 30 minutes.

IV.

The Influence of the Size of the Particles of Powdered Gelatine upon the Salt Action.

The fact that the after effect of a treatment with neutral salts with monovalent cation is the same for pig's bladder and for powdered gelatine, while it is different for gelatine in the form of one solid block, suggests that we are dealing with a surface effect of the salt. In order to test this suggestion powdered gelatine of four different sizes of particles was prepared, by sifting Cooper's powdered gelatine through sieves with different openings, namely, with 50, 60, 80, 100, and 120 wires per inch. The first lot contained particles which went through 50, but not through 60 (designated 50–60); the next those going through Sieve 60 but not through No. 80 (designated 60–80), and so on. 2 gm. of each of these particles were put into a funnel as described and first 25 cc. of M/8 NaCl were poured on each lot. In order to insure equal action of the salt the solution was kept in contact with the gelatine for ½ hour before it was allowed to filter. The swelling was measured and then 25 cc. of distilled water were poured on the gelatine and the H_2O was kept in contact with the gelatine for 20 minutes before it was allowed to filter through, and the swelling was measured again. This was followed by again pouring 25 cc. of distilled water into each funnel, keeping the H_2O in contact with the gelatine for 45 minutes, then allowing it to run through, and then again measuring the swelling. Table XI gives the result.

TABLE XI.

	Swelling of powdered gelatine of different grain size. Swelling measured in mm. height of the cylindrical mass of gelatine.			
	Size of particles.			
	50–60	60–80	80–100	100–120
Swelling after 25 cc. M/8 NaCl had been allowed to percolate	23	25	24	23
Additional swelling after first 25 cc. H_2O had been allowed to percolate	3	6	8.5	10
Additional swelling after the second 25 cc. H_2O had been allowed to percolate	19	27	31	38

It is obvious that the smaller the particles the greater the retention of water and the greater the swelling. This would be expected if the after effect of the salt upon the swelling in distilled water were a surface effect; for the total surface of a given mass of gelatine is of course the greater the smaller the size of the particles.

It should be possible to calculate the increase in surface with the decrease of the size of the powdered granules if the phenomenon were not complicated by a second variable which acts in the opposite sense as the size of the granules and which the writer believes to be the process of packing. The "packing" diminishes the free area of the particles.

The excessive swelling of pig's bladder under the influence of a previous treatment of NaCl is therefore a phenomenon which can be repeated in powdered gelatine but not in solid blocks of gelatine, and hence must be due to a difference in the structure of the two groups of systems, the gelatine block being homogeneous with only a comparatively small outer surface while the pig's bladder and the mass of powdered gelatine or ovomucoid consist of small discrete elements with an enormous internal surface.

The writer is inclined to believe that the salts combine with the gelatine and as a consequence modify the chemical affinity of the surface of the discrete particles for water. The result is a greater retention of water after the free salt solution has been replaced by distilled water. The gelatine or ovomucoid salts with univalent cations retain the water in such cases very powerfully, while the colloidal Ca salts do not possess this peculiarity. The mechanism of the swelling of powdered colloids or animal membranes like pig's bladder in distilled water, after a previous treatment with a neutral salt with univalent cation, is different from the mechanism of swelling of a solid block of gelatine under the influence of acid or alkali. The latter case has been explained very elegantly by Procter.[8]

[8] Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313. Procter, H. R., and Wilson, J. A., *ibid.*, 1916, cix, 307.

V.

The Antagonistic Salt Action on the Percolation of Water through Powdered Colloids.

In 1905 the writer[9] suggested that the antagonism between salts with monovalent and bivalent cations was due to the fact that small quantities of bivalent cations prevented the diffusion of the salts with univalent cations through the animal membranes. This idea has since been generally accepted and has received support by the work of many experimenters, especially the brilliant experiments of Osterhout[10] on *Laminaria*. It seemed, therefore, of interest to see in which sense the salts with univalent and bivalent cations influence the rate of diffusion or percolation of water through powdered colloids.

We must keep in mind that the antagonistic salt effects described in this paper differ in an essential point from the antagonistic salt effects on the living organism. The observations on the antagonistic action of salts on living organisms were all made while the living object was *in* the salt solution; and the only exceptions from this rule are the observations on washed eggs.[2] The antagonistic effects described in this paper deal with the behavior of colloids *after* the salt solution has been replaced by distilled water and after all the free salt solution has been washed away.

It is very easy to examine the influence of a salt treatment upon the rate of percolation of liquids through powdered colloids. 2 gm. of powdered gelatine or ovomucoid are put into a cylindrical funnel in the way described at the beginning and 25 cc. of a salt solution carefully poured on top of the mass. This solution runs through very rapidly as long as the particles are not too small. This is followed by pouring 25 cc. of distilled water upon the mass and this is then repeated. The rate of percolation becomes slower with each washing (due possibly to a denser packing of the particles) and after two or three washings with distilled water a definite effect of the previous salt treatment upon the rate of percolation can be discovered. This effect is exactly the reverse of the influence of the salts upon the subsequent swelling in

[9] Loeb, *Arch. ges. Physiol.*, 1905, cvii, 252.

[10] Osterhout, W. J. V., *Plant World*, 1913, xvi, 129; *Proc. Am. Phil. Soc.*, 1916, lv, 533.

distilled water. Thus a treatment of powdered gelatine with M/8 NaCl increases the rate of swelling of the mass in distilled water (after the salt solution is washed off) but it diminishes the rate of percolation of distilled water through the mass. $CaCl_2$ neither favors swelling nor does it retard the rate of percolation; it may accelerate it slightly.

It is thus easy to demonstrate an antagonistic salt action upon the rate of percolation of water through powdered gelatine. The same solutions as in Table VIII were used in the following experiment. 1 gm. of powdered gelatine was put into each funnel and at first the various mixtures of NaCl + $CaCl_2$ solutions (25 cc. in each case) were allowed to run through. This was followed by repeated washings with 25 cc. of distilled water. Table XII gives the cc. of water which percolated from the funnels into a measuring cylinder after 25 cc. of H_2O had been poured on the mass for the third time (third washing).

TABLE XII.

Cc. of H_2O which percolated in	Rate of percolation of 25 cc. of distilled water through powdered gelatine after a previous treatment with the following solutions and two washings with 25 cc. H_2O							
	Cc. M/8 $CaCl_2$ in 100 cc. M/8 NaCl							H_2O
	0	1	2	4	8	16	32	
159 min.	9	10.4	13.6	13.2	19.1	18.4	21.4	22.4
17 hrs.	23	24	26*	24	30*	26.5	25.5	27*

* Some of the water retained in previous washings had filtered through.

The rate of percolation is slowest in the gelatine previously treated with M/8 NaCl; and here the swelling is greatest. This is natural since the swelling as well as the lowering of the rate of percolation have the same cause, namely, the retention of water by the powdered gelatine. The same experiment can be made with powdered ovomucoid.

The writer has not yet tried any experiments on the influence of a previous salt treatment on the diffusion of water through a membrane of pig's bladder, though he intends to do so.

The experiments mentioned here bear a certain resemblance to the observations of soil chemists on the percolation of water through soil previously treated with salts. The writer's attention

was called to this work by Professor Lipman in Berkeley, in whose laboratory the subject has recently been investigated by Mr. Sharp.[11] It seems that many years ago A. Meyer first observed the fact that if soil had been soaked with certain salts it became impermeable for water, after the salt had been leached out. Schlösing and Van Bemmelen showed that the phenomenon was connected with a greater degree of suspensibility of the soil after such a treatment.[12] Soil treated with M/8 NaCl becomes almost impermeable for water after the salt solution is washed out. No measurable swelling of the soil follows when the soil is first treated with M/8 NaCl, then with distilled water until all the salt solution is driven out, and the soil becomes highly impermeable. The impermeability in this case is much greater than in the case of powdered gelatine or ovomucoid and the suspicion is justified that the impermeability of the soil after a treatment with NaCl is at least partly due to a denser packing of the particles. This variable may also be at least partly responsible for the retardation of percolation of water through powdered gelatine after a previous treatment with NaCl.

$CaCl_2$ does not retard the subsequent percolation of water through soil and it is easy to demonstrate the antagonism between NaCl and $CaCl_2$ upon the subsequent rate of percolation of water through soil after the salt is leached out.

10 gm. of finely powdered garden soil were put into each of a series of cylindrical funnels. Then 25 cc. of the antagonistic salt mixtures were poured on the soil and the time measured until 20 cc. of the solution had diffused into a measuring cylinder put under the funnel. Then 25 cc. of H_2O were poured into each funnel and again the time for 20 cc. of liquid to run through the soil was measured, and this was repeated three times. Table XIII gives the results.

It may be possible to make practical use of this action of Ca (which seems to be the same for all bivalent cations) for rendering impermeable soil permeable for water.

The writer does not wish to enter into the cause of this behavior of soil beyond mentioning that if all organic matter of the soil is

[11] Sharp, L. T., *Proc. Nat. Acad. Sc.*, 1915, i, 563; *Univ. Cal. Publ. in Agricult. Sc.*, 1916, i, 291.

[12] Van Bemmelen, J. M., *J. prakt. Chem.*, 1881, xxiii, 388

TABLE XIII.

	Time in minutes for 20 cc. of liquid to run through 10 gm. of soil in a cylindrical funnel.							
	Cc. M/8 $CaCl_2$ in 100 cc. M/8 NaCl.							Control H_2O
	0	1	2	4	8	16	32	
25 cc. salt.......	34	37.5	35	30.5	33	33	34	43
25 " H_2O......	73.5	66	50	46.5	40	34	33.5	44
25 " "	1,185*	1,185	1,185	1,185		66.5	43	68.5

* *I.e.*, over night.

destroyed by ignition a treatment of such soil with NaCl will no longer call forth the striking inhibition of the percolation of H_2O after the salt is leached out; but that an addition of some finely powdered organic colloid (powdered dry oak leaves, gum tragacanth, powdered gelatine, or ovomucoid) can restore to some extent this effect of a previous washing with NaCl. A mixture of finely powdered marble and powdered colloids acts like a mixture of ignited soil and organic colloids.

SUMMARY OF RESULTS.

1. Dried pig's bladder, freed from fat, when treated for a short time with a solution of a salt with univalent cation swells considerably more when subsequently put into distilled water, than it does if it remains permanently in the same salt solution or when it remains permanently in distilled water without a previous salt treatment.

2. It is assumed that this increased swelling of the membrane in distilled water after a previous treatment with one of the salts with univalent cation is due to an interaction between the salt and a constituent (probably protein) of the membrane; when the bladder remains permanently in the salt solution the latter prevents the swelling which takes place as soon as the salt solution is replaced by H_2O or a very weak salt solution.

3. A treatment of the membrane with salts with a bivalent cation (Mg, Ca, Sr, and Ba) does not induce the excessive swelling when the membrane is subsequently exposed to distilled water. Neither does such a treatment induce a dehydration of

the membrane. Membranes previously treated with salts with a bivalent cation swell when afterwards put into distilled water approximately to the same extent as membranes that have not been treated with any salt.

4. The addition of about 8 cc. of M/8 $CaCl_2$ to 100 cc. of M/8 NaC prevents the after effect which a treatment with a pure M/8 NaCl solution produces. It should be noticed that $CaCl_2$ does not influence swelling in the opposite sense from that of NaCl, but that it renders the after effect of the treatment with NaCl impossible in some other way.

5. It is impossible to repeat these effects of a previous salt treatment upon the subsequent swelling in distilled water with solid blocks of gelatine, or with sheets of gelatine.

6. It is, however, possible to repeat them with powdered gelatine or with powdered water-insoluble ovomucoid (and probably a large number of other powdered colloids).

7. The fact that pig's bladder behaves in regard to these phenomena like powdered colloids but not like solid blocks or sheets of gelatine suggests that the salt effects described in this paper are due to an action upon the surface of colloidal particles (fibers in the case of pig's bladder).

8. This suggestion is supported by the fact that the effect of a previous treatment with M/8 NaCl upon the subsequent swelling of a given mass of powdered gelatine in distilled water is greater when the size of the particles is smaller and hence the total internal surface greater.

9. It follows from all this that the mechanism of the swelling described in this paper is of a different nature from that observed in solid masses of gelatine under the influence of acid or alkali.

10. Observations upon the rate of percolation of water show that the effect of salt upon the subsequent rate of percolation of distilled water through the powdered gelatine varies inversely with the rate of swelling. A previous treatment with M/8 NaCl solution retards the percolation of water through the powdered gelatine, while a previous treatment of the mass with M/8 $CaCl_2$ has no such effect. The addition of a small quantity of $CaCl_2$ to NaCl prevents the subsequent retardation of the rate of percolation of water as it prevents the swelling.

11. It has been known that a treatment of soil with NaCl

renders the soil almost impermeable to water after the salt is leached out. In this case, however, no swelling of the soil seems to take place and the writer is not certain whether the influence of a salt treatment upon the percolation of water through powdered gelatine and ovomucoid is identical with or only analogous to that upon the percolation of water through soil.

THE METABOLISM OF SULFUR.

II. THE INFLUENCE OF SMALL AMOUNTS OF CYSTINE ON THE BALANCE OF NITROGEN IN DOGS MAINTAINED ON A LOW PROTEIN DIET.*

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Recent studies of the metabolism of protein with the aid of the more accurate methods of analysis, developed largely by Folin and by Van Slyke and their coworkers, have shown that amino-acids are absorbed as such from the alimentary tract, and circulate in the blood or are stored in the tissues. This has led to a realization of the fact that the rôle of the proteins in nutrition is a function of their component amino-acids, and that the adequacy or inadequacy of any individual protein is dependent upon the quantitative relationships of the amino-acids of its molecule. Attention accordingly has been centered on the problem of the ability of the organism to synthesize or dispense with the various individual amino-acids.

Certain proteins, such as gelatin, have long been known to be lacking in specific amino-acids.

Other proteins not completely lacking in any essential amino-acid may yet contain some of these amino-acids in the molecule in such small amounts as to render a high protein intake necessary to supply a sufficient quantity of the requisite amino-acids. This point of view has been clearly defined by Osborne and Mendel (1) in their studies on the growth requirements of the white rat. These observers have shown that growth on diets containing different proteins in approximately the same percentage relation may vary considerably, provided the percentage of protein in the diet is near the minimum requirement for these animals. On a diet containing a higher

* I wish to express my indebtedness to Professor A. E. Taylor of the University of Pennsylvania for courtesies which have facilitated the continuance of this research at the University of Illinois.

percentage of protein, these variations in nutritive efficiency are less marked or disappear altogether. They have also for certain proteins demonstrated the specific amino-acids the quantity of which determines the minimum for that protein. Thus rats failed to grow at a normal rate on diets containing less than 15 per cent of casein, while the addition of cystine to food containing 9 per cent of casein without further change at once rendered the diet decidedly more adequate for growth. Similar results were obtained with edestin to which lysine was added, and later (2) with lysine and gliadin. Recently by similar methods of study Hogan (3) has shown that for the proteins of the corn kernel, tryptophane is the first limiting factor and lysine the second.

Of the amino-acids, tyrosine (or phenylalanine (4)), tryptophane, lysine, and cystine are generally recognized as essential amino-acids which must be present preformed in adequate amounts in the diet. Ackroyd and Hopkins (5) have recently reported experiments which indicate that either histidine or arginine but not both must be present in the diet for normal nutrition. The claim of the indispensability of cystine preformed in the diet is based largely on indirect evidence or analogy, rather than on direct experimental evidence. With the exception of the work of Osborne and Mendel, in which the addition of cystine to casein, a protein notably low in cystine, was demonstrated to lower the percentage of casein required for normal growth, little experimental work on this point is available. The high cystine content of the proteins of the epithelial tissue, the constant loss of this sulfur-rich protein through the hair, skin, etc., especially in the lower mammals, and the inability to use inorganic sulfates for protein synthesis and growth have led to a belief in the indispensability of preformed cystine in the diet. Practical feeding experiments with swine (6) have also furnished evidence of a possible rôle of cystine. The offspring of sows fed during pregnancy with blood albumin (a protein rich in sulfur and in which a large part of the sulfur is presumably present as cystine) were larger, with heavier and darker coats of hair than the controls. McCollum and Davis (7) have suggested that the loss of sulfur from casein when heated may be associated with the decreased efficiency of such casein as a foodstuff. Holt in a recent address (8) has made the suggestion that the success attained in infant nutrition with whole milk from cows may be due not to the higher percentage of protein as such in whole milk, but to the fact that by thus increasing the percentage of protein in the diet, the infant's actual amino-acid needs for growth, especially in lysine and cystine, may be more nearly satisfied. Abderhalden (9) attempted without success to remove the cystine from completely hydrolyzed protein by precipitation with glacial acetic acid in order to determine the "*biologische Wertigkeit*" of cystine by feeding the resultant product to animals. In acceptance of this belief in the indipensability of cystine, many workers have added cystine to the products of hydrolysis of proteins before feeding, to replace the cystine destroyed in the course of the hydrolysis (Abderhalden (4) and Totani (4), Geiling (5)).

The experiments reported in the present communication represent an attempt to approach the problem of the cystine (and sulfur) requirement of the organism of the dog from the standpoint of the protein minimum, making use of variations in the nitrogen balances as indications of any change in the adequacy of the diet.

The general plan of the experiments has been as follows. Dogs were maintained on standard diets of low protein content but of ample calorific value. After control periods on these diets, small amounts of cystine (0.5 to 1.0 gm. of cystine daily) were added to the standard diets, and the influence of the cystine was noted as evidenced by changes in the nitrogen balances. In certain experiments as additional controls, nitrogen in the form of glycocoll, a dispensable amino-acid, and of tyrosine, an essential amino-acid, was added to the standard diet in amount equivalent to that added in the form of cystine. The animals used were females, and were accustomed to laboratory conditions, one of the animals (Dog A) having been in the writer's possession for over 3 years, the subject of frequent nutrition experiments. The animals were kept in the usual metabolism cages and the urine was separated into 24 hour periods by daily catheterization. There was no evidence of cystitis at any time. Separation of the feces into periods was accomplished by the use of carmine. In order to give consistency and bulk to the feces, neutral calcium phosphate (Merck's "Blue Label") was added to the diet, the pure calcium phosphate being chosen rather than the usual bone ash in order to avoid as far as possible the presence of sulfur in the diet other than the sulfur of the protein. The source of the protein of the diet was beef heart, trimmed from the adjacent fat, finely ground, and carefully mixed to ensure uniformity. In one experiment (Dog G), it was necessary to substitute for the beef heart, finely chopped steak from which connective tissue and fat had been removed as far as possible before grinding. The cane sugar, lard, and starch were pure commercial preparations. The purity of the cystine, which was prepared from wool by acid hydrolysis according to Folin, was established by analyses for nitrogen and sulfur.

Nitrogen was determined by the Kjeldahl-Gunning method. For the determination of sulfur in the food a weighed sample was evaporated to dryness in a porcelain evaporating dish with concentrated nitric acid on the water bath, the dried residue treated with Benedict's copper nitrate-potassium chlorate oxidation mixture, evaporated, and treated as in the method for urine.

The standard daily diets are shown in Table I. In the experiments with Dog A, Series I, and Dogs C and G, the cystine was added without alteration of the diet. In the experiments with Dog A, Series II, and Dog B, in order to keep the nitrogen intake constant, when the amino-acids were fed, an equivalent amount of nitrogen in the form of beef heart was removed from the diet. The starch was first made into a smooth paste with water, the other ingredients were added, and the whole was thoroughly mixed.

TABLE I.

Standard Diets.

	Dog A.		Dog B.	Dog C.	Dog G.
	Series I.	Series II.			
Sucrose, *gm.*	100	50	90	90	70
Starch, *gm*	40	30	20	40	40
Lard, *gm*	50	40	50	50	50
Calcium phosphate, *gm*	5	5	5	5	10
Water, *cc*	400	400	450	450	500
Total calories*	1,010	680	890	970	890
Calories per kg. body weight**	61	42	74	74	59

* This figure does not include the calories received in the form of beef heart. These varied slightly in individual periods, but approximated 100 calories in all but the experiments with Dog A, Series I, in which the calorific value approximated 125 calories.

** Exclusive of the calories from the beef heart.

The main difficulty experienced in the experiments was a refusal of the food after the experimental diet had been fed for some days. The diets contained much non-nitrogenous material and little meat, so that the mixed food had little to offer in the way of palatability. Although by forced feeding it would no doubt have been possible to prolong the experiments, we have felt that the lack of appetite which would make this necessary, and the inability by forced feeding to administer the food absolutely quantitatively, would render the results of little value. For this reason the two experiments with Dog A were most successful. During two periods of 35 and 42 days respectively, this animal consumed the food completely within 30 minutes after feeding. Dog C refused the diet at the end of 18 days and vomited the food after forced feeding, necessitating a termination of the experiment. Dog G took the diet well up to the 16th day. Forced feeding of a part of the food became necessary on this and on the succeeding day. For this reason the experiment was discontinued.

The details of the experiments are shown in Tables IV to VIII. In every case the addition of small amounts of cystine (equivalent to 0.058 to 0.116 gm. of N) to the low protein diet favorably influenced the nitrogen balance, while the addition of equivalent amounts of nitrogen in the form of glycocoll (Tables VI and VIII) exerted no such influence. The results obtained with tyrosine and phenylalanine (Tables VI and VII) indicate that under the conditions of the experiment these substances had little if any influence on the state of nitrogenous equilibrium, at least no effect

in any way comparable with that of cystine. Thus in Table VI, the addition of 0.75 gm. of cystine to the diet lowered the negative balance from −7.54 gm. for a 6 day period to −2.98 gm., a change from a daily average of −1.26 to −0.50 gm. On the withdrawal of the cystine, the negative balance increased to −5.49 gm. or −0.92 gm. daily, and remained practically constant at −5.64 and −5.44 gm. after the addition of glycocoll and tyrosine in the two following periods. In the next period addition of cystine

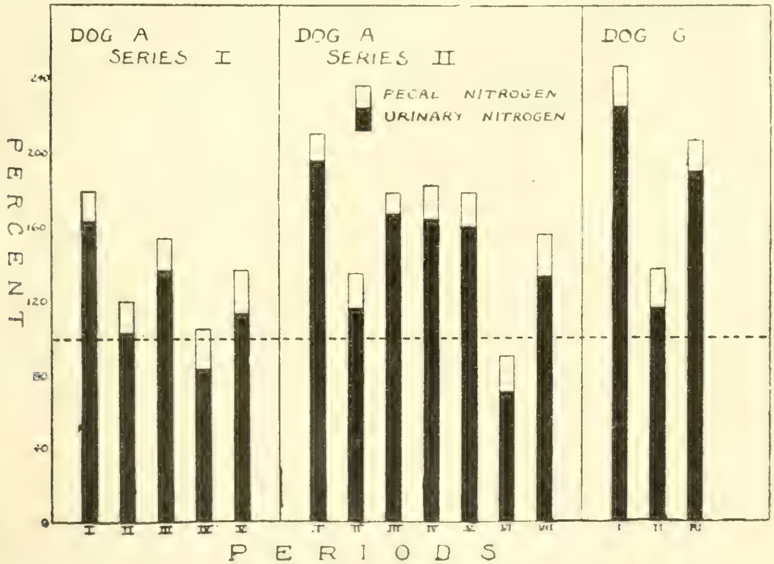

FIG. 1. The nitrogen eliminations are expressed in terms of the percentage of the dietary nitrogen (100 per cent, dotted line). The percentages are computed from the figures in Table II, which should be consulted for an explanation of the diet of the various periods.

resulted in a *positive* nitrogen balance of +0.45 gm. for the period, or +0.07 gm. daily, followed by a negative balance of −3.28 gm. in the subsequent period on removal of the cystine again from the diet. If the 1st day of each period, the transitional day, be omitted from the averages, the changes in the balances become more striking. Table II represents the average daily nitrogen eliminations and balances of the various experiments with the 1st day of each period omitted from the average. From the data in this

table, the excretion of urinary and fecal nitrogen and the nitrogen balances have been computed in terms of the percentage of dietary nitrogen, and the results plotted (Fig. 1) for three of the experiments. The dotted line represents 100 per cent or the nitrogen intake. All that portion of the chart above the dotted line represents the negative balance in terms of the percentage of the intake of nitrogen.

TABLE II.

Animal.	Experiment No.	Nitrogen.				Period.
		Diet.	Urine.	Feces.	Balance.	
		gm.	*gm.*	*gm.*	*gm.*	
Dog A, Series I.	I	1.48	2.41	0.23	−1.16	Control.
	II	1.57	**1.62**	0.26	**−0.31**	**Cystine.**
	III	1.55	2.12	0.27	−0.84	Control.
	IV	1.53	**1.29**	0.32	**−0.08**	**Cystine.**
	V	1.39	1.58	0.32	−0.51	Control.
Dog B.	I	1.22	1.97	0.24	−0.99	Control.
	II	1.22	1.69	0.21	−0.68	Tyrosine.
	III	1.22	**1.26**	0.24	**−0.28**	**Cystine.**
Dog C.	I	1.27	**1.73**	0.28	**−0.74**	**Cystine.**
	II	1.08	2.59	0.38	−1.89	Control.
Dog A, Series II.	I	1.14	2.24	0.16	−1.26	Control.
	II	1.14	**1.32**	0.22	**−0.30**	**Cystine.**
	III	1.14	1.90	0.24	−1.00	Control.
	IV	1.14	1.86	0.22	−0.94	Glycocoll.
	V	1.14	1.82	0.21	−0.89	Tyrosine.
	VI	1.14	**0.81**	0.22	**+0.11**	**Cystine.**
	VII	1.14	1.53	0.25	−0.64	Control.
Dog G.	I	1.28	2.88	0.37	−1.97	Control.
	II	1.54	**1.78**	0.32	**−0.56**	**Cystine.**
	III	1.54	2.92	0.26	−1.64	Glycocoll.

The failure of glycocoll to influence the loss of body nitrogen is of importance in view of the results of Abderhalden (4) which show that the addition of glycocoll or alanine to the diet of mice on a non-protein diet causes a diminished loss of nitrogen from the organism. It might be argued that the effect of cystine is due to a similar action, but the fact that glycocoll and tyrosine when fed

under the same experimental conditions failed to produce a like decrease in the loss of nitrogen from the body speaks against this explanation. We believe that we are dealing with a specific demand for cystine in an animal maintained on a low protein diet, that this diet is adequate or nearly so in its nitrogen content, but that it is deficient in cystine, which is the amino-acid that determines the minimum protein requirements in this case. It is worthy of note that the most successful experiments were obtained with long haired animals, Dogs A and G.

TABLE III.

	Beef heart.	Cat muscle.
	per cent	*per cent*
Total protein	15.01	
" " (N × 6.25)	17.56	
" N	2.81	
" protein N	2.39	2.92
" sulfur	0.180	0.213
" protein S	0.110	0.132
Per cent of N as protein	85.0	
" " " S " "	60.1	61.5*
N:S	15.6	
" (protein)	21.72	22.1*

* Calculated from the figures of Lee, Scott, and Colvin (10).

Concerning the nature and amount of the protein sulfur in the diet fed in these experiments, *i.e.*, beef heart, little information is available. As shown in the tables the diet was low in sulfur as well as in nitrogen. Analyses of the beef heart showed a ratio of total nitrogen to sulfur of 14 – 16 : 1, which is similar to that usually accepted as a general figure for pure proteins. However, considerable amounts of both nitrogen and sulfur are present in beef heart as non-protein extractives so that the above ratio does not necessarily represent the true relationship between protein nitrogen and sulfur. We have, therefore, analyzed the beef heart mixture as fed in these experiments in order to ascertain the relationship between the protein nitrogen and sulfur of the diet under consideration. Analyses of this nature are reported by Lee, Scott, and Colvin (10) for certain muscles of the cat, but no similar analyses of beef heart muscle could be found in the litera-

ture. The protein content of the mixture was determined by the method of Janney (11) and the resultant preparation analyzed for nitrogen and sulfur. The analytical results are presented in Table III, with the figures of Lee, Scott, and Colvin included for purposes of comparison. The results obtained show that the percentage of extractive sulfur in the beef heart is greater than is the percentage of extractive nitrogen; that is, the protein sulfur is lower in proportion to the total sulfur than is protein nitrogen in proportion to the total nitrogen. The N : S ratio of the protein is 21.7 as compared with 15.6, the ratio of the whole beef heart muscle. The protein, therefore, fed in the present series of experiments was lower in its sulfur content than the ordinary proteins of the diet. No attempt was made to determine what proportion of this protein sulfur was present as cystine sulfur. The accumulation of further data on the sulfur and cystine content of various muscles is desirable.

SUMMARY.

The addition of small amounts of cystine to the diet of dogs on a low protein diet diminished the loss of nitrogen from the body and favorably influenced the nitrogen balance. This is interpreted to be the result of a specific demand for cystine for metabolic purposes, since tyrosine and glycocoll added to the diet under like conditions of experimentation did not diminish the nitrogen loss or influence the condition of nitrogenous equilibrium.

TABLE IV.

Dog A. Black Long Haired Female.

Period.	Day.	Weight.	Intake. Nitrogen.	Intake. Sulfur.	Urinary nitrogen.	Fecal nitrogen.	Nitrogen balance.	Diet.
		kg.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
I	1	16.59	1.48	0.096	2.77	0.23	−1.52	Standard diet and 50 gm. beef heart daily.
	2	16.56	1.48	0.096	2.39	0.23	−1.14	
	3	16.55	1.48	0.096	2.43	0.23	−1.18	
	4	16.54	1.48	0.096	2.39	0.23	−1.14	
	5	16.54	1.48	0.096	2.39	0.23	−1.14	
	6	16.51	1.48	0.096	2.39	0.23	−1.14	
	7	16.54	1.48	0.096	2.44	0.23	−1.19	
Total			10.36	0.672	17.20	1.61	−8.45	
Average			**1.48**	**0.096**	**2.46**	**0.23**	**−1.21**	
II	8	16.57	1.57	0.363	2.08	0.26	−0.77	As Period I plus 1.0 gm. cystine.
	9	16.57	1.57	0.363	1.73	0.26	−0.42	
	10	16.61	1.57	0.363	1.62	0.26	−0.31	
	11	16.57	1.57	0.363	1.65	0.26	−0.34	
	12	16.61	1.57	0.363	1.57	0.26	−0.26	
	13	16.72	1.57	0.363	1.52	0.26	−0.21	
	14	16.76	1.57	0.363	1.62	0.26	−0.31	
Total			10.99	2.541	11.79	1.82	−2.62	
Average			**1.57**	**0.363**	**1.68**	**0.26**	**−0.37**	
III	15	16.80	1.55	0.096	1.73	0.27	−0.45	As Period I.
	16	16.75	1.55	0.096	2.09	0.27	−0.81	
	17	16.67	1.55	0.096	2.05	0.27	−0.77	
	18	16.70	1.55	0.096	2.03	0.27	−0.75	
	19	16.70	1.55	0.096	2.05	0.27	−0.77	
	20	16.71	1.55	0.096	2.19	0.27	−0.91	
	21	16.74	1.55	0.096	2.31	0.27	−1.03	
Total			10.85	0.672	14.45	1.89	−5.49	
Average			**1.55**	**0.096**	**2.06**	**0.27**	**−0.78**	
IV	22	16.74	1.57	0.230	2.03	0.32	−0.78	As Period I plus 0.5 gm. cystine.
	23	16.75	1.57	0.230	1.26	0.32	−0.01	
	24	16.75	1.57	0.230	1.32	0.32	−0.07	
	25	16.80	1.57	0.230	1.25	0.32	−0.00	
	26	16.79	1.57	0.230	1.30	0.32	−0.05	
	27	16.85	1.44	0.230	1.28	0.32	−0.16	
	28	16.93	1.44	0.230	1.34	0.32	−0.22	
Total			10.73	1.610	9.78	2.24	−1.29	
Average			**1.53**	**0.230**	**1.40**	**0.32**	**−0.18**	

TABLE IV—*Concluded.*

Period.	Day.	Weight.	Intake.		Urinary nitrogen.	Fecal nitrogen.	Nitrogen balance.	Diet.
			Nitrogen.	Sulfur.				
		kg.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
V	29	16.97	1.39	0.096	1.42	0.32	−0.35	As Period I.
	30	16.92	1.39	0.096	1.54	0.32	−0.47	
	31	16.89	1.39	0.096	1.63	0.32	−0.56	
	32	16.96	1.39	0.096	1.58	0.32	−0.51	
	33	16.92	1.39	0.096	1.61	0.32	−0.54	
	34	16.93	1.39	0.096	1.55	0.32	−0.48	
	35	16.99	1.39	0.096	1.56	0.32	−0.49	
Total			9.73	0.672	10.89	2.24	−3.40	
Average			**1.39**	**0.096**	**1.56**	**0.32**	**−0.49**	

TABLE V.

Dog C. White Short Haired Female.

Period.	Day.	Weight.	Intake.		Urinary nitrogen.	Fecal nitrogen.	Nitrogen balance.	Diet
			Nitrogen.	Sulfur.				
		kg.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
I	1	13.54	1.27	0.211	1.62	0.28	− 0.63	Standard diet, 40 gm. beef heart and 0.5 gm. cystine.
	2	13.47	1.27	0.211	1.57	0.28	− 0.57	
	3	13.47	1.27	0.211	1.82	0.28	− 0.83	
	4	13.39	1.27	0.211	1.62	0.28	− 0.63	
	5	13.34	1.27	0.211	1.59	0.28	− 0.60	
	6	13.37	1.27	0.211	2.07	0.28	− 1.08	
Total			7.62	1.266	10.29	1.68	− 4.34	
Average			**1.27**	**0.211**	**1.72**	**0.28**	**− 0.72**	
II	7	13.22	1.21	0.077	3.18	0.38	− 2.35	Standard diet and 40 gm. beef heart.
	8	13.01	1.08	0.077	2.52	0.38	− 1.82	
	9	13.02	1.08	0.077	2.64	0.38	− 1.94	
	10	13.06	1.08	0.077	2.71	0.38	− 2.01	
	11	13.04	1.08	0.077	2.39	0.38	− 1.69	
	12	13.01	1.08	0.077	2.68	0.38	− 1.98	
Total			6.61	0.462	6.12	2.28	−11.79	
Average			**1.10**	**0.077**	**2.69**	**0.38**	**− 1.97**	
III	13	13.04	1.14	0.211	1.69			As Period I.
	14	12.97	1.14	0.211	1.50			

Animal refused to eat; experiment discontinued.

TABLE VI.

Dog A. Weight 15 Kg.

Period.	Day.	Intake. Nitrogen.	Intake. Sulfur.	Urinary nitrogen.	Fecal nitrogen.	Nitrogen balance.	Diet.
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	
I	1	1.14	0.082	2.21	0.16	−1.23	Standard diet and 40 gm. beef heart.
	2	1.14	0.082	2.06	0.16	−1.08	
	3	1.14	0.082	2.20	0.16	−1.22	
	4	1.14	0.082	2.40	0.16	−1.42	
	5	1.14	0.082	2.22	0.16	−1.24	
	6	1.14	0.082	2.33	0.16	−1.35	
Total		6.84	0.492	13.42	0.96	−7.54	
Average		**1.14**	**0.082**	**2.24**	**0.16**	**−1.26**	
II	7	1.14	0.276	1.92	0.22	−1.00	Standard diet, 37 gm. beef heart, and 0.75 gm. cystine.
	8	1.14	0.276	1.46	0.22	−0.54	
	9	1.14	0.276	1.41	0.22	−0.49	
	10	1.14	0.276	1.33	0.22	−0.41	
	11	1.14	0.276	1.21	0.22	−0.29	
	12	1.14	0.276	1.17	0.22	−0.25	
Total		6.84	1.656	8.50	1.32	−2.98	
Average		**1.14**	**0.276**	**1.42**	**0.22**	**−0.50**	
III	13	1.14	0.082	1.38	0.24	−0.48	As Period I.
	14	1.14	0.082	1.85	0.24	−0.95	
	15	1.14	0.082	1.94	0.24	−1.04	
	16	1.14	0.082	1.94	0.24	−1.04	
	17	1.14	0.082	1.88	0.24	−0.98	
	18	1.14	0.082	1.90	0.24	−1.00	
Total		6.84	0.492	10.89	1.44	−5.49	
Average		**1.14**	**0.082**	**1.82**	**0.24**	**−0.92**	
IV	19	1.14	0.076	1.84	0.22	−0.92	Standard diet, 37 gm. beef heart, and 0.47 gm. glycocoll.
	20	1.14	0.076	1.85	0.22	−0.93	
	21	1.14	0.076	1.87	0.22	−0.95	
	22	1.14	0.076	1.87	0.22	−0.95	
	23	1.14	0.076	1.86	0.22	−0.94	
	24	1.14	0.076	1.87	0.22	−0.95	
Total		6.84	0.456	11.16	1.32	−5.64	
Average		**1.14**	**0.076**	**1.86**	**0.22**	**−0.94**	

TABLE VI—*Concluded.*

Period.	Day.	Intake. Nitrogen.	Intake. Sulfur.	Urinary nitrogen.	Fecal nitrogen.	Nitrogen balance.	Diet.
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	
V	25	1.14	0.076	1.94	0.21	−1.01	Standard diet, 37 gm. beef heart, and 1.12 gm. tyrosine.
	26	1.14	0.076	1.94	0.21	−1.01	
	27	1.14	0.076	1.91	0.21	−0.98	
	28	1.14	0.076	1.86	0.21	−0.93	
	29	1.14	0.076	1.69	0.21	−0.76	
	30	1.14	0.076	1.68	0.21	−0.75	
Total		6.84	0.456	11.02	1.26	−5.44	
Average		**1.14**	**0.076**	**1.84**	**0.21**	**−0.91**	
VI	31	1.14	0.276	1.03	0.22	−0.11	As Period II.
	32	1.14	0.276	0.81	0.22	+0.11	
	33	1.14	0.276	0.70	0.22	+0.22	
	34	1.14	0.276	0.75	0.22	+0.17	
	35	1.14	0.276	0.83	0.22	+0.09	
	36	1.14	0.276	0.95	0.22	−0.03	
Total		6.84	1.656	5.07	1.32	+0.45	
Average		**1.14**	**0.276**	**0.85**	**0.22**	**+0.07**	
VII	37	1.14	0.082	0.96	0.25	−0.07	As Period I.
	38	1.14	0.082	1.27	0.25	−0.38	
	39	1.14	0.082	1.55	0.25	−0.66	
	40	1.14	0.082	1.59	0.25	−0.70	
	41	1.14	0.082	1.66	0.25	−0.77	
	42	1.14	0.082	1.59	0.25	−0.70	
Total		6.84	0.492	8.62	1.50	−3.28	
Average		**1.14**	**0.082**	**1.44**	**0.25**	**−0.55**	

TABLE VII.

Dog. B. Short Haired White Female Bull Dog.

Period.	Day.	Weight.	Nitrogen intake.	Urinary nitrogen.	Fecal nitrogen.	Nitrogen balance.	Diet.
		kg.	*gm.*	*gm.*	*gm.*	*gm.*	
I	1	12.36	1.22	1.99	0.24	−1.01	43 gm. beef heart and standard diet.
	2	12.29	1.22	1.91	0.24	−0.93	
	3	12.23	1.22	2.11	0.24	−1.13	
	4	12.20	1.22	1.95	0.24	−0.97	
	5	12.21	1.22	1.83	0.24	−0.85	
	6	12.10	1.22	2.05	0.24	−1.07	
Total			7.32	11.84	1.44	−5.96	
Average			**1.22**	**1.97**	**0.24**	**−0.99**	
II	7	12.00	1.22	1.82	0.21	−0.81	40 gm. beef heart plus 0.9 gm. phenylalanine and standard diet.
	8	12.04	1.22	1.39	0.21	−0.38	
	9	12.00	1.22	1.87	0.21	−0.86	
	10	11.89	1.22	1.69	0.21	−0.69	1.0 gm. tyrosine substituted for phenylalanine.
	11	11.91	1.22	1.68	0.21	−0.68	
	12	11.98	1.22	1.80	0.21	−0.80	
Total			7.32	10.25	1.26	−4.22	
Average			**1.22**	**1.71**	**0.21**	**−0.70**	
III	13	11.90	1.22	1.55	0.24	−0.57	40 gm. beef heart, 0.7 gm. cystine, and standard diet.
	14	11.88	1.22	1.30	0.24	−0.32	
	15	11.82	1.22	1.32	0.24	−0.34	
	16	11.88	1.22	1.27	0.24	−0.29	
	17	11.93	1.22	1.10	0.24	−0.12	
	18	11.93	1.22	1.14	0.24	−0.16	
Total			7.32	7.68	1.44	−1.80	
Average			**1.22**	**1.28**	**0.24**	**−0.30**	

TABLE VIII.

Dog G. Long Haired Collie. Weight 15 Kg.

Period.	Day.	Nitrogen intake.	Urinary nitrogen.	Fecal nitrogen.	Nitrogen balance.	Diet.
		gm.	*gm.*	*gm.*	*gm.*	
I	1	1.16	3.27	0.37	−2.48	Standard diet plus beef heart 40 gm.
	2	1.16	3.04	0.37	−2.25	
	3	1.16	2.89	0.37	−2.10	
	4	1.16	2.88	0.37	−2.09	
	5	1.45	2.81	0.37	−1.73	Hamburg steak 43 gm.
	6	1.45	2.79	0.37	−1.71	
Total		7.54	17.68	2.22	−12.36	
Average		**1.26**	**2.95**	**0.37**	**−2.06**	
II	7	1.54	2.44	0.32	−1.22	Standard diet, Hamburg steak 43 gm., plus cystine 0.75 gm.
	8	1.54	1.74	0.32	−0.52	
	9	1.54	1.67	0.32	−0.45	
	10	1.54	1.67	0.32	−0.45	
	11	1.54	1.86	0.32	−0.64	
	12	1.54	1.94	0.32	−0.72	
Total		9.24	11.32	1.92	−4.00	
Average		**1.54**	**1.89**	**0.32**	**−0.67**	
III	13	1.54	1.97	0.26	−0.69	Standard diet, Hamburg steak 43 gm., plus glycocoll 0.45 gm.
	14	1.54	2.67	0.26	−1.39	
	15	1.54	3.11	0.26	−1.83	
	16	1.54	2.92	0.26	−1.64	
	17	1.54	2.96	0.26	−1.68	
Total		7.70	13.63	1.30	−7.23	
Average		**1.54**	**2.73**	**0.26**	**−1.45**	

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NUTRITION INVESTIGATIONS UPON COTTONSEED MEAL.

III. COTTONSEED FLOUR. THE NATURE OF ITS GROWTH-PROMOTING SUBSTANCES, AND A STUDY IN PROTEIN MINIMUM.

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In the first of a series of studies upon the nutritive value of cottonseed flour,[1] we reported:

> "Our results indicate that cottonseed meal does not contain sufficient mineral for growth, is not actively toxic, contains efficient protein, and perhaps fat-soluble growth-promoting substances, similar to those of butter fat, but in less adequate quantities."

A second paper[2] further demonstrates the efficiency of the protein of cottonseed flour for the normal growth, development, and reproduction of the albino rat. It is the purpose of this paper to consider the content in cottonseed flour of growth-essential factors other than protein and mineral, and to report the results of studies of the protein minimum of cottonseed flour.

A preceding report showed that when rats received 50 per cent cottonseed flour in a diet containing no additional growth-promoting substances other than those furnished by cottonseed flour, considerable growth was experienced for 135 to 205 days and that when the amount of cottonseed flour was increased to 70 per cent with no additional growth-promoting substances there has been normal growth for 90 days and continuous growth for 165 to 205 days. These results clearly indicate that 50 per cent cottonseed flour furnishes a considerable amount of growth-pro-

[1] Richardson, A. E., and Green, H. S., *J. Biol. Chem.*, 1916, xxv, 307.

[2] Richardson and Green, *J. Biol. Chem.*, 1917, xxx, 243.

moting substances although probably not enough for normal growth, whereas 70 per cent of the flour furnishes an amount sufficient at least for normal growth during 90 days in spite of the fact that this diet is decidedly deficient in mineral content.[3] However, from these experiments we could draw no conclusions as to whether this growth was due to the fat-soluble or water-soluble growth factor, or to both, or deduce anything as to the relative amounts of these two substances.

We reported also that 50 per cent fat-free, ether-extracted flour in a diet made up with lard and starch failed to induce as pronounced growth or maintain animals without complete failure for as long a period as did the unextracted flour, due probably to the absence of the ether-soluble, growth-essential substance. However, animals grew for 145 to 190 days and then maintained weights on the ether-extracted flour for 240 days, which seems to indicate in the light of the results reported in the present paper that there must be present in the ether-extracted flour a considerable amount of the water-soluble growth-promoting factor.

A series of carefully controlled experimental diets designed to throw light on the relative amounts of fat-soluble and water-soluble growth factors in cottonseed flour has been fed for several weeks.

Composition of Diets.

		per cent	*per cent*
A.	Ether extract cottonseed flour	4.35	12
	Casein	18.	18
	Lard	17.65	10
	Mineral Mixture III	5	5
	Starch	30	30
	Lactose	25	25

[3] Ash analysis of cottonseed flour (Golaz).

	per cen
SiO_2	0.14
Cl	None.
SO_3	0.06
P_2O_5	2.57
K_2O	2.01
CaO	0.26
MgO	0.25
Na_2O	None.

B.	Ether extract cottonseed flour	4.35	12
	Lard	17.65	10
	Casein	18	18
	Mineral Mixture III	5	5
	Starch and water extract of cottonseed flour	20	20
	Starch	35	35
C.	Casein	18	
	Butter fat	12	
	Lard	12	
	Mineral Mixture III	5	
	Starch and water extract of cottonseed flour	20	
	Starch	33	
D.	Casein	18	
	Lard	24	
	Mineral Mixture III	5	
	Starch	53	

The casein used in these diets is purified according to the method described by McCollum and Davis.[4] The mineral employed in the rations is supplied by Mineral Mixture III, described by the same authors.[5]

Mineral Mixture III.

	per cent
NaCl	12.31
K_2HPO_3	28.08
$CaH_4(PO_4)_2H_2O$	0.74
$MgSO_4$ (anhydrous)	1.56
Mg citrate	5.75
Na " (anhydrous)	3.12
Ca lactate	46.80
Fe "	1.64

In Diet A the water-soluble growth factor is supplied by lactose of the purity of ordinary reagents.[6]

Diets A and B receive the fat-soluble growth factor contained in the ether extract of cottonseed flour. The extract is made by percolating the flour until the ether comes through colorless. The ether is then driven from the extract by evaporation over a hot

[4] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 233.

[5] McCollum and Davis, *J. Biol. Chem.*, 1915, xxi, 625.

[6] McCollum and Davis, *J. Biol. Chem.*, 1915, xxiii, 181.

water bath at 60°C. These two diets were first made up with 4.35 per cent of the ether extract, an amount equivalent to 50 per cent flour in the diet. After 11 days the amount was increased to 12 per cent to enable a comparison of the relative amounts of fat-soluble growth-promoting substance contained in butter fat and in cottonseed flour fat.

Diets B and C receive the water-soluble growth factor contained in the water extract of cottonseed flour dried on starch. The cottonseed flour is extracted for 1 hour with frequent stirring with ten times its weight of distilled water. The liquid is filtered free from the flour, acidified with dilute acetic acid, and heated to boiling to coagulate any possible soluble protein. It is then filtered, evaporated down at 60°C., and dried on starch over a water bath at 60°C. with the aid of electric fans. The water extract of 800 gm. of cottonseed flour dried on 200 gm. of starch weighs 120 gm., the weight of both the extract and starch being 320 gm. Therefore 1 gm. of the starch plus extract is equivalent to 2.5 gm. of cottonseed flour, and 20 per cent of this substance in the diet is equivalent to 50 per cent cottonseed flour, which is the amount of flour generally employed in our experimental rations previously discussed.

Diet C receives the fat-soluble growth factor from butter fat.

From Chart 1 it may be seen that stock rats growing normally at the age of 36 days when placed upon Diet A supplying the water-soluble growth-promoting substance from lactose, and containing only 4.35 per cent ether extract of cottonseed flour, equivalent to 50 per cent flour in the diet, do not receive sufficient fat-soluble, growth-promoting substance from this amount of ether extract to continue normal growth. When the amount of ether extract is increased to 12 per cent, comparable to the content of butter fat which induces normal growth and well-being of the animal, all other nutritive factors being favorable, these same animals although retarded in growth during 11 days on the diet containing 4.35 per cent ether extract now resume a normal rate of growth.

When animals receive in their diet the fat-soluble accessory of butter fat and the water-soluble accessory of cottonseed flour equivalent to 50 per cent flour in the diet, as in Diet C, it will be seen from Chart 1 that rats grow perfectly normally.

When both the water-soluble and fat-soluble substances are supplied from 20 per cent water extract and 12 per cent ether extract respectively, of cottonseed flour, it will be seen from Chart 1 that rats grow normally although their growth was retarded during 11 days by the insufficient amount of fat-soluble growth factor supplied by only 4.35 per cent ether extract.

Chart I indicates the behavior of animals on a diet deficient in both the water-soluble and fat-soluble growth-promoting substances. When placed on Diet D the growth of these animals is immediately retarded and after 4 weeks, they begin to lose in weight.

These results indicate that 50 per cent cottonseed flour in a diet furnishes sufficient water-soluble accessory for normal growth, but does not furnish enough fat-soluble accessory. As compared with 12 per cent butter fat, 12 per cent ether extract of cottonseed flour equivalent to 138 per cent of flour is apparently as efficient in supplying the fat-soluble food accessory.

Earlier studies of cottonseed flour[1,2] reported the efficiency, both for growth and normal reproduction of the albino rat, of diets in which cottonseed flour furnished the only source of protein. These diets furnished an abundance of protein, 25 per cent, as well as the other essentials to normal growth of the rat. To test still further the efficiency of cottonseed proteins as compared with those proteins of known physiological value, a series of experiments was made to determine the protein minimum of cottonseed flour.

The experimental diets contain all the essentials to growth with varying amounts of protein, 4 to 18 per cent as follows.

		per cent
23.	Cottonseed flour	36
	Protein-free milk	10
	Butter fat	12
	Lard	16
	Starch	26
24.	Cottonseed flour	24
	Protein-free milk	10
	Butter fat	12
	Lard	16
	Starch	38

44.	Cottonseed flour	8
	Butter fat	12
	Lard	15
	Protein-free milk	22
	Starch	43
45.	Cottonseed flour	18
	Butter fat	12
	Protein-free milk	22
	Lard	15
	Starch	33
46.	Cottonseed flour	12
	Butter fat	12
	Lard	15
	Protein-free milk	22
	Starch	37

On a diet in which 18 per cent protein is furnished by 36 per cent cottonseed flour as in Diet 23, rats have grown practically normally and are still alive at the age of 410 days. As may be seen from Chart II, the females have grown larger than the average female, whereas the males have been very slightly under size. Three of the four females on this diet have reproduced, Rat 1027 giving birth to three litters, and Rat 1029 to two litters. Of the six families produced on this diet only six young, Nos. 2060–5, from two families, have survived. At the age of 148 days the one male rat is a little above four-fifths of the average weight at that age, while at the age of 159 days the females are slightly under average weight.

When 12 per cent protein is furnished by 24 per cent cottonseed flour as in Diet 24 the majority of the animals have not grown normally (Chart III) although Female 1028 attained normal full growth quicker than the average individual. Only one of three females ever reproduced, No. 262, giving birth to three females, Nos. 2029, 2030, 2031, all of which are alive at the age of 269 days, fine appearing animals but below average size.

On Diet 45 furnishing only 9 per cent protein from cottonseed flour, all the animals are decidedly under weight at the age of 165 days. But Female 310 at the age of 155 days gave birth to eight fine looking young, all of which she devoured (Chart IV). These results do not altogether agree with the work reported by

Osborne and Mendel.[7] They obtained normal growth with 9 per cent cottonseed protein although they report no reproduction.

Similarly, as seen by Chart IV, on Diet 46 which furnishes only 6 per cent cottonseed protein our rats have been able to show very little growth during 108 days, the animals averaging only 8 to 9 gm. increase in weight, whereas Osborne and Mendel have obtained considerable growth on 6 per cent cottonseed protein.

With Diet 44 containing only 4 per cent protein, an amount smaller than any considered by Osborne and Mendel, rats have behaved as indicated by Chart IV. When first placed on this diet there is a decided loss in weight for several days, after which there is an almost successful attempt at maintenance for about 50 days. These animals are still continued on this diet and though stunted are in good condition, extremely active, and have fine coats of fur.

SUMMARY.

1. 20 per cent of the water extract of cottonseed flour dried on starch, equivalent per gm. to 2.5 gm. of cottonseed flour, *i.e.*, 50 per cent cottonseed flour in the diet, contains sufficient water-soluble food accessory for normal growth.

2. 4.35 per cent of the ether extract of cottonseed flour equivalent per gm. to 11.5 gm. of cottonseed flour, *i.e.*, 50 per cent cottonseed flour in the diet, does not contain sufficient fat-soluble food accessory for normal growth, but 12 per cent of the ether extract appears quite as efficient in supplying enough of the fat-soluble accessory for normal growth as does an equivalent amount of butter fat.

3. 18 per cent cottonseed protein when supplied with adequate amounts of all other necessary nutritive factors induces practically normal growth of the male rat, and better than average growth in the female, and fairly normal reproduction, with high mortality among the second generation. At the age of 148 days the male of the second generation is about four-fifths average size and the female slightly under size.

4. 12 per cent cottonseed protein does not induce perfectly normal growth. On this diet one female has borne three young,

[7] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxix, 289.

all of which are alive at the age of 269 days, although below average size.

5. Normal growth has not been obtained on 9 per cent cottonseed protein but at the age of 155 days one animal, No. 310, has borne a fine looking litter of eight young, all of which she devoured.

6. Very little growth has been obtained with 6 per cent protein, the average gain in weight during 108 days being 8 to 9 gm.

7. With only 4 per cent cottonseed protein rats have fallen off in weight when first placed upon this diet but have almost successfully maintained their weight for 50 days thereafter.

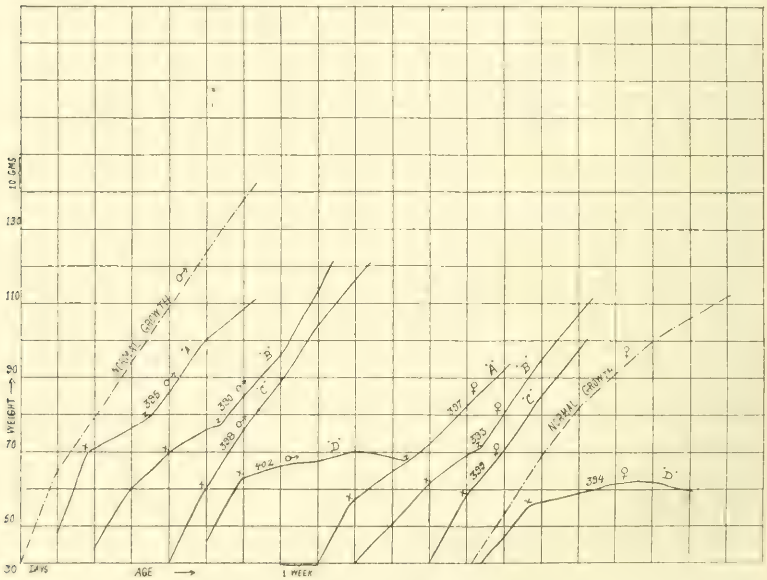

CHART I. X indicates the point at which the rats were given the experimental diets. With animals on diets A and B, XZ indicates the period during which the diet contained 4.35 per cent ether extract of cottonseed flour. Z indicates the point at which the amount of ether extract was increased to 12 per cent. Rats receiving 20 per cent water extract of cottonseed flour with all other nutritive factors favorable in Diet C grow normally. Rats receiving 12 per cent ether extract of cottonseed flour with all the nutritive factors favorable in Diet A, period YZ, grow practically normally. Rats receiving both water and ether extracts of cottonseed flour in Diet B, period Z, grow normally. Rats on Diet D lacking in both water-soluble and fat-soluble food accessories do not continue normal growth.

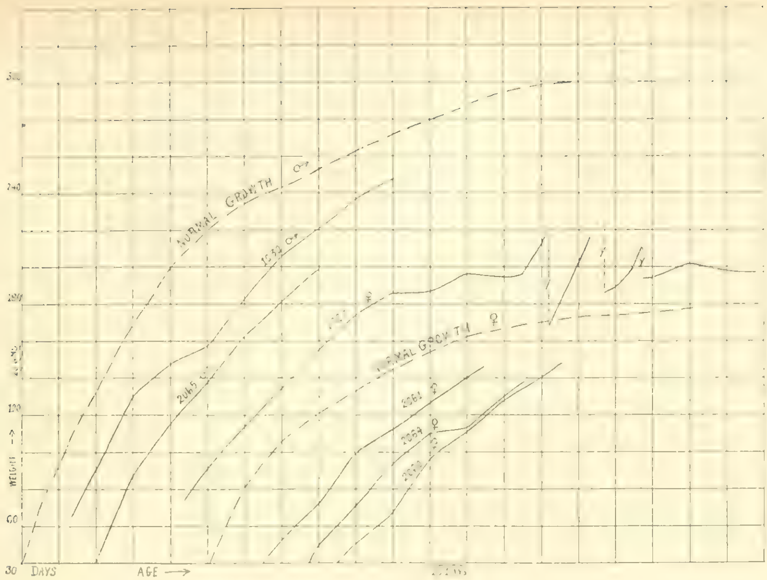

CHART II. Indicates the behavior of animals on Diet 23, furnishing 18 per cent cottonseed protein. Rats 2060, 2061, 2064, and 2065 are of the second generation on this diet raised since weaning on Diet 23.

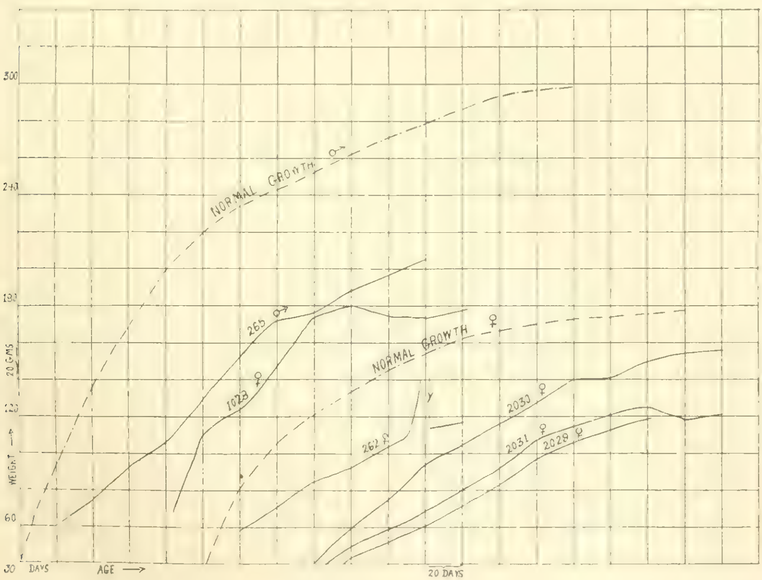

CHART III. Indicates the behavior of animals on Diet 24, furnishing 12 per cent cottonseed protein. Rats 2029, 2030, and 2031 are of the second generation on this diet raised since weaning on Diet 24.

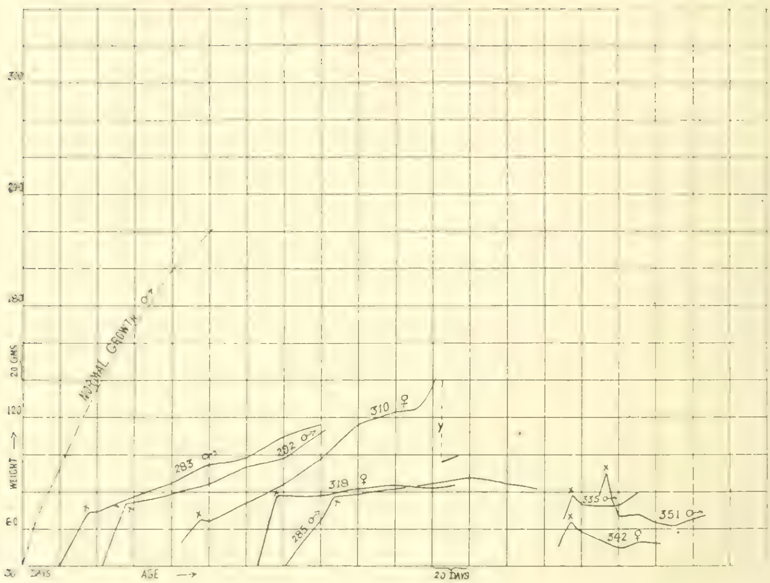

CHART IV. The curves of Rats 283 and 292 indicate the behavior of animals receiving 9 per cent cottonseed protein in Diet 45. Rats 318 and 285 have received 6 per cent cottonseed protein in Diet 46. Rats 335, 342, and 351 received only 4 per cent cottonseed protein in Diet 44.

THE AVAILABILITY OF THE ENERGY OF FOOD FOR GROWTH.*

By C. R. MOULTON.

(*From the Department of Agricultural Chemistry of the University of Missouri, Columbia.*)

(Received for publication, June 4, 1917.)

Food which enters the animal body has a certain amount of total energy, called the heat of combustion. This energy is different for different feeds. Of this total energy the animal loses part by way of the feces in the undigested food residues, part by way of the urine in incompletely oxidized bodies, and part by way of combustible gases voided. The amount over and above these losses is called the metabolizable energy. Not all of this metabolizable energy is available for the uses of the animal body in either maintenance or growth. There is a loss due to the work of digestion, mastication, and movement of the food through the digestive tract. There is also a further loss due to a stimulated metabolism upon the absorption of digestible substances from the alimentary tract. There may be a slightly greater muscular activity due to the increased food consumed. All this energy is converted into heat and lost from the body. What is left of the metabolizable energy after these second losses are accounted for is called the net, or available, energy. This may be used for production of work, or may be stored in the animal body in the form of protein, fat, or other body substances.

In connection with the general "Use of Food Experiment" conducted at the University of Missouri Agricultural Experiment Station since 1907, some data have been obtained upon the relative amount of the energy of the food which may be recovered in flesh gained. The animals used were mature beef steers, 2 or 3 years old, of the Shorthorn breed. They were as nearly alike in body weight, previous method of treatment, and type as it is possible to select beef steers. One animal was somewhat heavier than the other two. The ration used in the work consisted of five parts of mixed grain to two parts of alfalfa hay. The mixed grain

* Read before the Division of Biological Chemistry of the American Chemical Society, April 12, 1917.

was eight parts maize meal (corn chop) and one part old process linseed meal.

The digestibility of the ration was determined by digestion trials. The cost of maintaining the animals at constant weight was determined by extended maintenance trials. Greater details will be found in previously published work.[1] After the maintenance trial one animal, Steer 18, was slaughtered for analysis. Two other animals were fattened, one to full prime condition, Steer 48, and the other to a condition 40 to 50 days under prime, Steer 121, when they were slaughtered and analyzed (Table I).

TABLE I.

Composition of Animals.

	Weight.		
	Steer 18.	Steer 121.	Steer 48.
Warm empty weight, *gm*	302,183	508,513	744,708
Per cent of water	57.34	50.02	41.73
Weight of water, *gm*	173,259	254,339	310,750
Per cent of fat	18.03	29.72	41.25
Weight of fat, *gm*	54,479	151,131	307,164
Per cent of nitrogen	2.96	2.51	2.07
Weight of nitrogen, *gm*	8,955	12,776	15,391
Weight of protein, *gm*	55,968	79,847	96,194
Per cent of ash	5.70	4.14	3.45
Weight of ash, *gm*	17,211	21,064	25,697
Per cent of phosphorus	1.07	0.74	0.62
Weight of phosphorus, *gm*	3,230	3,769	4,648

Since Steer 121 during maintenance, or at the beginning of the full fed period, weighed less than the check animal and Steer 48 weighed more it is necessary to calculate their composition assuming the same percentage composition as the check animal had. Table II shows the results together with the calculations for the composition of the gain.

In calculating the thermal equivalent of the fat and protein gained it was necessary to use the data of other investigators. For protein the value of 5.6776 calories per gm. was used. This

[1] Trowbridge, P. F., Moulton, C. R., and Haigh, L. D., *Missouri Agric. Exp. Station Research Bull. 18*, 1915.

is the value found by Köhler[2] for the lean muscular tissue of beef cattle from which the fat had been removed by ether and a correction made for the fat in the residue as determined by the Dormeyer[3] method. For fat the value of 9.4889 calories per gm. was used. This is the average of four results for beef fat quoted by Fries,[4] namely, those of Stohman and Langbein, Stohman and associates, Gibson, and Danilewsky.

Steer 121 stored up 926,359 calories in the fat gained and 141,252 calories in the protein gained. This is a total of 1,067,611 calories. Steer 48 stored up 2,355,601 calories in fat gained and

TABLE II.

Composition of Gain. Energy Stored in Flesh Gained.

	Steer 121.		Steer 48.	
	Flesh gained (estimated).	Composition of gain (estimated).	Flesh gained (estimated).	Composition of gain (estimated).
	gm.	*per cent*	*gm.*	*per cent*
Warm empty weight	211,726	.....	417,900	.....
Water	84,174	39.76	123,372	29.52
Fat	97,626	46.11	248,246	59.40
Nitrogen	3,981	1.88	5,707	1.37
Protein	24,879	11.75	35,667	8.54
Ash	4,161	1.97	7,084	1.70
Phosphorus	597	0.28	1,155	0.28
Energy in fat, *calories*	926,359		2,355,601	
" " protein, "	141,252		202,502	
Total energy stored, "	1,067,611		2,558,103	

202,502 calories in protein gained. This is a total of 2,558,103 calories.

In order to make these gains and store this energy these animals consumed a large amount of feed. Steer 121 consumed over 2,000 pounds of digestible organic nutrients and Steer 48 consumed nearly 8,000 pounds. The equivalent metabolizable energy was found by the method of Armsby.[5] For this ration it is 3,803

[2] Köhler, A., *Z. physiol. Chem.*, 1900–01, xxxi, 479.

[3] Dormeyer, C., *Arch. ges. Physiol.*, 1896–97, lxv, 102.

[4] Fries, J. A., *U. S. Dept. Agric., Bureau Animal Industry, Bull. 94*, 1907, 13.

[5] Armsby, H. P., and Fries, J. A., *J. Agric. Research*, 1914–15, iii, 451.

calories per kg. of digestible organic matter, or 1.72 therms per pound. A therm is 1,000 large calories. Table III gives the data.

The metabolizable energy that may be used for production of flesh is that amount over and above the needs for maintenance. Using the average weight of the animal while on maintenance and the maintenance cost found by trial for each, the cost of maintenance during the full feed period was calculated. The amounts

TABLE III.

Gross and Net Cost of Gain. Per Cent Availability of Energy.

	Steer 121.	Steer 48.
Length of period, *days*	153	567
Weight at beginning, *lbs*	764	842
" " end, *lbs*	1,266	1,805
" gained, *lbs*	502	963
Grain eaten daily, *lbs*	18.34	16.93
Hay eaten daily, *lbs*	7.21	7.01
Organic nutrients, *lbs*	3,398	11,821
Digestible organic nutrients, *lbs*	2,267	7,860
Metabolizable energy, *therms*	3,900	13,519
Energy per pound gain, *therms*	7.77	14.03
Average weight of animal, *lbs*	1,041	1,384
Energy per 1,000 pounds for maintenance, *therms*	12.14	12.73
Total energy for maintenance, *therms*	1,900	8,646
Energy above maintenance, *therms*	2,000	4,873
Energy above maintenance per pound of gain, *therms*	3.98	5.06
Energy recovered in gain, *therms*	1,067.6	2,558.1
Metabolizable energy recovered, *per cent*	53.39	52.49

of energy required for maintenance at different body weights are proportional to the body surfaces, that is, roughly to the two-thirds power of the body weights. The author has shown in previously published work[6] that the surface area of a thin or medium fleshed steer is more nearly proportional to the five-eighths power of the weight, while with very fat steers the five-ninths power should be used. For animals here discussed the five-ninths power was used.

[6] Moulton, C. R., *J. Biol. Chem.*, 1916, xxiv, 299.

According to the theory in the above paragraph the cost of maintenance for the thin animals, Steer 121 and Steer 48, should be proportional to the five-eighths power of the weights until they could be classed as fat and after that the five-ninths power should be used. A calculation was made using the extreme case of the five-eighths power entirely. This made a difference of 0.25 per cent of the net energy cost of a pound of gain for Steer 18 and 1.5 per cent for Steer 48 throughout the entire period. The true value would lie between the one given in Table III and a value smaller by the amount just shown. Therefore the error could hardly be more than one-half that shown, or about 0.75 per cent of the total amount for Steer 48. The error in the calculation of the per cent of available energy would be about double this error. The small size of the error involved makes it inadvisable to use a more complex method of calculation than that employed in the preceding paragraph.

The results of the calculations, given in Table III, show a much higher productive energy cost of each pound of gain for the very fat steer than for the medium fat steer. Table II shows that the gains of Steer 48 were about 29 per cent more fat than those of Steer 121. The productive energy consumed increased in about the same proportion (27 per cent) from 3.98 therms per pound to 5.06 therms.

In the tissue gained by these animals one recovered 53.39 per cent of the metabolizable energy consumed above maintenance and the other recovered 52.49 per cent. Thus it is seen that the very fat steer saved up almost as much of the energy above maintenance as did the medium fat steer. These figures average 52.94 per cent. Since this proportion of the energy is recovered it may be said that this is a measure of the availability and that the metabolizable energy of the ration here used is 52.94 per cent available, or net.

In the work of Armsby, previously referred to, animals were used similar to those here discussed and a ration—alfalfa hay and grain mixture No. 2—somewhat similar was used. Using the data given[7] the writer has calculated the availability. On an average 56.09 per cent of the metabolizable energy was available.

[7] Armsby and Fries,[5] pp. 443, 474.

Since the ration used by Armsby was richer in grain than the one used at the Missouri Agricultural Experiment Station it is advisable to calculate the availability from figures given elsewhere by Armsby. He shows[8] that alfalfa hay has 44 per cent of metabolizable and 17 per cent of net energy. This makes the net to be 38.636 per cent of the metabolizable. For grain mixture No. 2 he shows 65 per cent of metabolizable energy and 40 per cent of net energy. This makes the net energy to be 61.538 per cent of the metabolizable. Putting these two together in the ratio of five parts of grain to two parts of hay (the Missouri ratio) the value of 54.995 per cent is obtained. Armsby's figures show the ration to be 55 per cent available, while the energy stored by the steers used in the work discussed in this paper shows the ration to be about 53 per cent available. This is a remarkably close agreement and is an experimental verification of the work done by Armsby in his calorimeter.

[8] Armsby and Fries, *Penn. State College Agric. Exp. Station Bull. 142*, 1916, 13.

CONTRIBUTIONS TO THE CHEMICAL DIFFERENTIATION OF THE CENTRAL NERVOUS SYSTEM.

IV. THE RELATIVE AMOUNT OF SHEATHING SUBSTANCE IN THE CORPUS CALLOSUM AND INTRADURAL NERVE ROOTS (MAN AND DOG).

By W. KOCH and M. L. KOCH.

(From the Hull Laboratory of Biochemistry and Pharmacology, University of Chicago, The Wistar Institute of Anatomy and Biology, Philadelphia, and the Psychiatric Institute, Ward's Island, New York.)*

(Received for publication, June 18, 1917.)

A comparison of the chemical composition of the nerve fibers from the corpus callosum and from the lumbosacral intradural nerve roots in man and in the dog was undertaken in order to determine whether in these two mammals the relative amount of sheathing substance is the same in the two divisions of the nervous system, the callosum representing the central and the nerve roots the peripheral system.

By sheathing substance is meant the myelin sheath which surrounds the axis cylinder and which gives myelinated nerve fibers their glistening white appearance. The term "white matter" has been applied to groups of myelinated fibers as these appear in the

* In 1911–12 this work was begun at the University of Chicago, at the suggestion of my brother, Dr. Waldemar Koch, under a grant from the Wistar Institute of Anatomy and Biology. At this time the dog material was collected and analyzed, also the human corpus callosum, which was obtained through the kindness of Dr. H. Gideon Wells of the Department of Pathology. Dr. Koch died in 1912 and since then I have completed the work. The three samples of human intradural nerve roots were obtained through the kindness of Dr. G. H. Whipple of the Department of Pathology, Johns Hopkins Medical School. Into the period of my present position at the Psychiatric Institute falls the bringing together and working up of the results.

I wish to express my appreciation to Dr. H. H. Donaldson for his interest and aid in bringing these data into shape for publication.

M. L. K.

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central nervous system. Barring the insignificant longitudinal striae and the glia cells, the corpus callosum is purely white matter.

The lumbosacral intradural nerve roots, forming the cauda equina, emerge from the lower levels of the cord and run for some distance within the dura. These also are groups of myelinated fibers.

Both these parts represent white matter as distinguished from gray, but the callosal fibers are without a neurilemma and are intermingled with neuroglia elements, while the intradural nerve roots contain fibers with a neurilemma and are held together by connective tissue, which, nevertheless, is scanty in this locality.

TABLE I.

The Percentage of Water and of Ether-Alcohol Extract Based on the Weight of Dry Substance in the Myelinated Fibers of the Corpus Callosum and of the Lumbosacral Intradural Nerve Roots of the Mature Dog (Hatai).

Dog.	Corpus callosum.		Intradural nerve roots.	
	Water.	Extract.	Water.	Extract.
	per cent	*per cent*	*per cent*	*per cent*
Fat (old)	66.94	70.54	66.44	73.13
Nearly fat-free (old)	69.58	67.90	69.97	68.76
" "	70.21	67.37	70.45	68.36
Fat moderate	71.21	66.71	70.50	66.94
Average	69.58	68.13	69.34	69.29

Hatai[1] found in the dog a great similarity between the water content and the ether-alcohol extract of the corpus callosum on the one hand and of the intradural nerve roots on the other (Table I).

Our own observations on this point by a method like that used by Hatai, show similar volume relations not only in the dog but also in man (Table II) although the values for the intradural nerve roots of man deviate somewhat from those to be expected.

[1] Dr. Hatai's data have not been published heretofore, but he kindly permits us to use them in this paper. It is to be noted that the ether-alcohol extract obtained by Dr. Hatai is not exactly equivalent to the "extract fraction" of the latter tables as the latter includes besides the ether-alcohol extract the water extract.

TABLE II.

The Percentage of Water and of Ether-Alcohol Extract Based on the Weight of Dry Substance in the Myelinated Fibers of the Corpus Callosum and of the Lumbosacral Intradural Nerve Roots of the Mature Dog and Man (Koch).

Animal.	Corpus callosum.		Intradural nerve roots.	
	Water.	Extract.	Water.	Extract.
	per cent	*per cent*	*per cent*	*per cent*
Dog (average of two)............	70.11	69.21	69.32	71.46
Man " " "	70.31	70.75	72.18	64.71

These results of Hatai led Donaldson to conclude that the degree of myelination was probably very similar in these two parts of the nervous system. Assuming that the volume of the myelin in the myelinated fibers of the peripheral nerves is about equal to, or in some cases greater than, that of the axis cylinder,[2] then if the general chemical composition was the same it would follow that similar volume relations were true for the central fibers. If we should moreover find that the chemical constituents, especially those predominating in the sheath, were in corresponding proportions, the argument would be fairly complete that the relative amount of sheathing substance is the same or nearly the same in these two types of white matter.

Nothing is definitely known of the function of the myelin sheath although, according to Mathews' Physiological Chemistry (1915), it is probably nutritive. It has a definite chemical composition, however, as distinguished from gray matter (Table III, column C, cortex, and column F, corpus callosum).

The data show that the white matter (corpus callosum) is poorer in water, proteins, and extractives, and richer in lipoids as compared with the gray matter. All the lipoids which were determined, even the phosphatides, are present in greater amounts in the white matter than in the gray matter.

The white matter also undergoes greater changes in its chemical composition than the gray matter during the development of the nervous system (see Table III). Donaldson[3] has shown that during the post-natal development of the mammalian nervous

[2] Donaldson, H. H., and Hoke, G. W., *J. Comp. Neurol. and Psychol.*, 1905, xv, 1. Greenman, M. J., *J. Comp. Neurol.*, 1913, xxiii, 479; 1917, xxvii, 403.

[3] Donaldson, H. H., *J. Comp. Neurol.*, 1916, xxvi, 444.

TABLE III.

*Man. Corpus Callosum and Cortex at Different Ages. Proportions of Constituents in Percentage of Solids.**

	A.	B.	C.	D.	E.	F.
	Whole brain.	Cortex.**	Cortex.**	Corpus callosum.†		
Laboratory No....	13	14	15	W. 41	14	15
Age..............	6 weeks.	2 years.	19 years.	Full term fetus.	2 years.	19 years.
1.						
Water, *per cent*....	88.78	84.49	83.17	89.92	76.45	69.70
Solids, " "	11.22	15.51	16.83	10.08	23.56	30.30
2.						
Proteins...........	46.6	48.4	47.1	48.4	31.9	27.1
Extract...........	53.4	51.6	52.9	51.6	68.1	72.9
Extractives (organic extractives and inorganic constituents)............	20.3	15.8	15.4	18.2	9.1	6.3
Lipoids...........	33.1	35.8	37.5	33.4	59.0	66.5
3.						
Phosphatides......	24.2	24.7	23.7	23.12	26.3	31.00
Cerebrosides......	6.9	8.6	8.8	.....	17.2	16.60
Sulfatides.........	1.6	....	1.2	3.33	....‡	8.65
Cholesterol (by difference)......	(0.4)	(2.5)	(3.8)	.....	....	(10.25)

* Koch, W., and Mann, S. A., *J. Physiol.*, 1907, xxxvi, 1. Table III is somewhat modified from the original table to make it comparable with the other tables in the present paper. Data for corpus callosum of full term fetus are added.

** Collection of gray matter; see Koch, W., *Am. J. Physiol.*, 1904, xi, 303-329.

† Collection of white matter; see p. 401

‡ 0.5 as SO_4.

system (rat), the progressive diminution of the water content of the entire brain and of the spinal cord, which is preeminently a function of age, is due mainly to the accumulation of myelin, using the term myelin to designate the sheathing substance, while the cell bodies and their axons do not suffer any significant loss

of water. The white matter loses from 18 to 20 per cent of water, while, according to the admixture of myelinated fibers, the gray matter loses from 2 to 5 per cent of water from birth to maturity. This loss of water is accompanied by changes in the relative proportions of the proteins and extract (including lipoids and extractives). These proportions are changed most in the white matter (corpus callosum), while in the gray matter (cortex) the relative proportions are only slightly altered, the main change being in the relation of the lipoids to the extractives in the extract fraction (Table III).

When we compare the brains of fetal pigs (5 and 10 cm. in length) and the rat at birth—both of which represent gray matter[4] with adult nervous tissue, we find the total lipoid content to be only about one-half as great as at maturity, while certain lipoids, such as the cerebrosides, are entirely absent and the sulfatides are present only in small amount at this stage (Table IV). The phosphatides are present in fairly large amount. The cholesterol was not determined in this series although it is probably present in small amount as an analysis of the brains of fetal dogs indicates (3.76 per cent) (determined directly by the Windaus[5] digitonin method).

TABLE IV.

Lipoids in the Brain of Fetal Pigs and of Albino Rats at Birth, Compared with the Lipoids in the Brain of the Adult Albino Rat (in Percentage of Solids).

	Total lipoids.	Cerebrosides.	Sulfatides.	Phosphatides.
5 cm. fetal pig	21.35	0.00	0.92	15.41
10 " " "	23.43	0.00	0.90	15.62
Rat at birth	24.87	0.00	1.45	15.20
Adult rat	41.70	9.00	4.60	22.00

The great increase of lipoids shown in Table IV, an increase which takes place after about the 10th day of age in the rat, is due for the most part to the formation of the myelin sheaths.

In our chemical studies[6] on the progressive changes in the central nervous system during growth, which were followed in the

[4] Koch, M. L., *J. Biol. Chem.*, 1913, xiv, 267.

[5] Windaus, A., *Z. physiol. Chem.*, 1910, lv, 113.

[6] Koch, W., and Koch, M. L., *J. Biol. Chem.*, 1913, xv, 423.

albino rat, we found that the most marked and characteristic of these chemical changes which occur during and just after the appearance of the myelin sheath are largely noticeable in the proportions of the individual lipoids which appear coincident with myelination. For example the phosphatides—which are not very differently distributed between the cell body and nerve fiber as indicated by a comparison of cortex with callosum[7] and which are present before myelination—increase at a remarkable rate at the beginning of myelination[8] and as this process proceeds, sulfatides, cerebrosides, and cholesterol are elaborated and laid down. These therefore are found chiefly in the myelin sheath.

It was suggested by one of us (W. K.) that the sulfatides (lipoid sulfur), which have been shown to be present in only small amounts in embryonic non-myelinated nervous tissue and which appear to be closely associated with the development of the sheath, might serve as an index for the amount of myelinated fibers in a given brain or from different parts of the same brain. This suggestion requires further study.

Material and Methods.

The material for the present study was obtained from man and dogs. The dog material, which was analyzed first, was obtained from the animals used for the laboratory classes in Physiology at the University of Chicago during the Spring Quarters of 1911 and 1912. The collection of material for each sample covered a period of about 2 weeks, as it required a large number of dogs to furnish a sufficient amount for chemical analyses, especially in the case of the nerve roots. The material at each period of collection was weighed and then put immediately into alcohol and preserved according to the methods employed in the chemical analyses of nerve tissue.[9] The ages of the animals from which the material was collected were not known. We aimed, however, to take dogs of average size, usually full grown, as this made the collecting of the nerve roots less tedious. The human material

[7] Koch, W., *Am. J. Physiol.*, 1904, xi, 306.

[8] Koch and Koch,[6] p. 443.

[9] Koch, M. L., and Voegtlin, C., *Bull. Hyg. Lab., U. S. P. H. S.*, 103 1916, 67–82.

was collected in 1912 and 1914. The corpus callosum data represent duplicate analyses of one case (this was checked with other analyses made by Waldemar Koch,[1]) and the data for the intradural nerve roots represent three analyses of three individual cases.

Collection of Material.—The method employed for the collection of the corpus callosum in both man and dog was as follows: The cerebrum was divided into its two hemispheres, each hemisphere was laid with its mesial surface on a glass plate and then cut vertically into sections about 2 cm. thick. These sections were then laid on the glass plate and with a sharp scalpel the white matter was cut out, leaving a margin of white attached to the gray matter. These pieces of white matter were weighed to 0.001 gm. Care was taken to expose the material as little as possible to the air. It was therefore kept tightly covered with a glass bell jar and the collection was made as rapidly as possible.

The intradural nerve roots were collected in the following way: In the dog the spinal cord was exposed and without removing the cord from the body the lumbosacral nerve roots were cut away between their attachment to the cord and their passage through the dura. In man the entire cord with the nerve roots attached was removed from the body, with the dura intact. The dura was then opened, each nerve root picked up carefully with a pair of forceps, and cut between its two attachments. Care was taken to clip off any ganglion substance at the distal end of the nerve roots. Each nerve root was placed immediately in a glass stoppered weighing bottle and weighed to 0.001 gm. The method employed in the chemical analysis of this material is described in detail elsewhere.[9] [10] This method in turn is based on the methods worked out and elaborated by Waldemar Koch for the analysis of brain tissue.[11]

Chemical Findings.

A comparison of the corpus callosum and the intradural nerve roots of the dog confirms the earlier observations of Hatai (Table I) as to the consistency of the ratios between the percentage of water and ether-alcohol extract in these two types of white matter (Table II). In man the percentage of water and of extract

[10] Koch, W., and Mann, S. A., *J. Physiol.*, 1907, xxxvi, 1.

[11] Koch, W., *J. Am. Chem. Soc.*, 1910, xxxi, 1329.

in the corpus callosum is in agreement with that in the dog. In the nerve roots, however, we find in man that the water is several (three) points higher and the extract is several (seven) points lower than in the dog (Table II). In this connection it may be noted that the human case, No. 17 (column C, Table V) shows the widest departure from the mean. The more detailed analysis of the individual constituents also indicates the greatest departure from the average in No. 17. The averages for the nerve roots in man were therefore based on the two remaining cases.

If in man (Table V) the determinations for the callosum are taken as the standard and those for the nerve roots compared with them, we find that the *water* and *proteins* are higher in the nerve roots, the *extractives* (organic extractives and inorganic constituents) are very much lower, while the *lipoids*, on the other hand, are in agreement.[12] For the individual lipoids we find that the *phosphatides* are slightly higher in the nerve roots, the *sulfatides* appear to be in agreement and the *cholesterol* also. The *cerebrosides* could not be compared as the small samples of the nerve roots did not permit a complete analysis to be made.

The *distribution of the sulfur in per cent of total sulfur*, in the protein, lipoid, and water-soluble fractions shows the protein sulfur to be in close agreement, the lipoid sulfur to be slightly lower, and the water-soluble sulfur to be very much higher. The *distribution of the phosphorus in per cent of total phosphorus* in the same fractions shows the protein phosphorus to be lower, the lipoid phosphorus to be slightly higher, and the water-soluble phosphorus to be very much lower. The total sulfur and the total phosphorus are practically the same in both callosum and nerve roots.

[12] In making a comparison between the values given in the accompanying tables, a definite plan has been followed. Using as a standard the value to which a second value is referred, the percentage deviation has been determined in each instance.

Values which vary from the standard used:

By less than	10 per cent	are considered in	*agreement.*
From	10–20 "	" " "	slightly different.
From	20–30 "	" " "	different.
Above	30 "	" " "	very different.

The language of comparison is not always the same, but the distinctions noted above have been carefully maintained.

TABLE V.

Man. Corpus Callosum and Intradural Nerve Roots. Proportions of the Constituents in Percentage of Solids.

	A.	B.	C.	D.	E.	F.	G.
	Corpus callosum.		Intradural nerve roots.			Corpus callosum, averages.	Intradural nerve roots, averages of No. 26 and No. 30.
Laboratory No.	W. 42 I.	W. 42 II.	17	26	30		
Case No.	Normal.	Normal.	N. I.	N. II.	N. IV.		
Weight of sample, *gm*.	47.86	39.82	8.90	6.71	6.17		
1.							
Water, *per cent*	70.22	70.40	(72.93)	72.31	72.06	70.31	72.18
Solids, " "	29.78	29.60	(27.07)	27.69	27.94	29.69	27.82
2.							
Proteins	29.37	29.13	(38.26)	35.40	35.18	29.25	35.29
Extract	70.63	70.87	(61.74)	64.60	64.82	70.75	64.71
Extractives (organic extractives and inorganic constituents)	5.12	(5.12)	(3.21)	2.80	2.73	5.12	2.76
Lipoids	65.51	65.75	(58.53)	61.80	62.09	65.63	61.95
3.							
Phosphatides	27.78	27.48	(34.08)	35.80	33.92	27.63	34.86
Cerebrosides						(16.60)*	
Sulfatides	7.33	7.60	(5.64)	7.76	7.83	7.46	7.79
Cholesterol			(11.77)	10.64	12.41	(10.25)*	11.52
4.							
Total sulfur	0.49	0.51	(0.49)	0.53	0.57	0.50	0.55
" phosphorus	1.43	1.42	(1.55)	1.58	1.52	1.42	1.55
5.	Distribution of sulfur in percentage of total sulfur.						
Protein S	56.43	51.20	(63.60)	52.02	52.00	53.80	52.01
Lipoid S	29.91	34.90	(22.80)	29.08	27.27	32.40	28.17
Water-soluble S**	13.66	13.80	(13.60)	18.90	20.74	13.80	19.82
6.	Distribution of phosphorus in percentage of total phosphorus.						
Protein P	12.80	12.00	(10.2)	8.52	9.56	12.40	9.04
Lipoid P	75.00	76.68	(85.00)	87.56	86.70	75.80	87.12
Water-soluble P**	12.20	11.40	(4.76)	3.95	3.74	11.80	3.84

* See Table III, 3, F.

** Water-soluble S and P in this table and Tables VI, VII, and VIII represent the sulfur and phosphorus found in the extractives (2).

If in the dog (Table VI) the determinations for the callosum are taken as the standard and those for the nerve roots compared with them, then we find that the values for *water, proteins,* and *lipoids* are in agreement, those for the *extractives* (organic extractives and inorganic constituents) are, as in man, lower in the nerve roots. For the individual lipoids we find that the *phosphatides* are higher in the nerve roots and the *sulfatides* are slightly lower as in man. The cerebrosides and cholesterol were not determined and can therefore not be compared.

The *distribution of the sulfur in per cent of total sulfur,* in the protein, lipoid, and water-soluble fractions shows the protein sulfur and the water-soluble sulfur to be in close agreement, while the lipoid sulfur is slightly higher. The *distribution of the phosphorus in per cent of total phosphorus* in the same fractions shows the protein phosphorus to be lower, the lipoid phosphorus slightly higher, and the water-soluble phosphorus very much lower in the nerve roots as compared with the callosum. The total sulfur is slightly lower, while the total phosphorus is slightly higher in the nerve roots as compared with the callosum.

In order to determine from the foregoing data whether the relative amount of sheathing substance in the corpus callosum and in the intradural nerve roots in man and dog is similar we shall first have to compare the white matter from the corpus callosum of man with that of the dog and to make a similar comparison in the case of the nerve roots, and, second, we shall have to compare the white matter from the callosum (dog) with that from the nerve roots in each of these two forms.

1. When we compare (Table VII) the white matter in the callosum of man with that in the callosum of the dog, *taking the data for the dog as the standard,* we find that the agreement for the water and the individual constituents is close, except for the extractives (organic extractives and inorganic constituents), which are low, and the water-soluble sulfur, which is very much higher in man.

When we compare the white matter in the nerve roots in man with that in the nerve roots of the dog, again *taking the data for the dog as the standard,* we find the agreement less close for the water as well as for the individual constituents. In the intradural nerve roots of man, both the water and the protein are

TABLE VI.

Dog. Corpus Callosum and Intradural Nerve Roots. Proportions of the Constituents in Percentage of Solids.

	A.	B.	C.	D.	E.	F.
	Corpus callosum.		Intradural nerve roots.		Corpus callosum, averages.	Intradural nerve roots, averages.
Laboratory No.	C. 100(a).	C. 100(b).	C. 101(a).	C. 101(b).		
Year of analysis	1911	1912	1911	1912		
No. of dogs used	10	8	19	18		
Weight of sample, *gm*	10.61	14.17	7.85	8.69		
1.						
Water, *per cent*	70.03	70.19	70.0	68.65	70.11	69.32
Solids, " "	29.97	29.81	30.0	31.35	29.89	30.68
2.						
Proteins	31.74	29.85	31.63	25.46	30.79	28.54
Extract	68.26	70.15	68.37	74.54	69.21	71.46
Extractives (organic extractives and inorganic constituents)	6.01	6.47	4.55	4.51	6.25	4.53
Lipoids	62.25	63.68	63.82	70.03	62.96	66.93
3.*						
Phosphatides	30.35	29.60	35.48	38.94	29.97	37.21
Sulfatides	8.22	8.72	7.26	7.84	8.47	7.53
4.						
Total sulfur	0.51	0.49	0.44	0.37	0.50	0.41
" phosphorus	1.54	1.51	1.64	1.77	1.52	1.70
5.	Distribution of sulfur in percentage of total sulfur.					
Protein S	57.92	55.90	56.32	49.78	56.91	53.05
Lipoid S	32.10	35.30	32.60	42.42	33.70	37.51
Water-soluble S	9.98	8.80	11.08	7.79	9.39	9.43
6.	Distribution of phosphorus in percentage of total phosphorus.					
Protein P	11.60	12.96	10.30	8.00	12.28	9.15
Lipoid P	76.22	75.67	83.75	85.29	75.94	84.52
Water-soluble P	12.18	11.37	5.95	6.71	11.77	6.33

* Samples were too small to permit cerebrosides and cholesterol to be determined.

again bring out, what has already been indicated above, that the chemical composition of the nerve roots of man does not appear to be as close to the callosum as is that of the nerve roots of the dog.

TABLE VIII.

Ratio of the Water-Soluble Sulfur to Protein Sulfur in the Corpus Callosum and Intradural Nerve Roots in Man and Dog, Also in the 2 Year Old Corpus Callosum in Man.

	Dog.		Man.		Man (2 yrs.)* corpus callosum.
	Corpus callosum.	Nerve roots.	Corpus callosum.	Nerve roots.	
Protein S	56.91	53.05	53.81	52.01	63
Water-soluble S	12.28	9.15	13.80	19.82	31
Ratios	1:4	1:5.6	1:4	1:2.6	1:2

* Koch.[10]

DISCUSSION.

To interpret the foregoing results it is necessary to make a general survey of them—and for this purpose we shall use the percentage value of the extract fraction, *i.e.*, the sum of the percentage values for the lipoids plus those for the extractives (organic extractives and inorganic constituents).

For the callosum in man we have (Table V, 2, A, B) 70.63 and 70.87 per cent—the results of two analyses on the same material. From an earlier study (Table III, 2, F) the data for man at 19 years gives 72.9 per cent.

For the callosum in the dog the corresponding values (Table VI, 2, A, B) are 68.26 and 70.15 per cent—the analyses being made on different groups a year apart; Hatai had found 68.13 per cent (Table I), but, as noted, Hatai's ether-alcohol extract should not be expected to give quite so large a value as appears in our extract fraction as the latter contains only part of the extractives (water-soluble) as these were only incompletely extracted by Hatai's method.

Reserving comment on these results for the callosum we pass to the corresponding data for the intradural nerve roots.

In the case of man (Table V, 2, C, D, E) the values for the extract from the nerve roots in three different cases were 61.74,

64.60, and 64.82 per cent, the first determination being distinctly lower than the other two and not used in making the averages. In the dog, on the other hand, the values are 68.37 and 74.54 per cent (Table VI, 2, C, D) while Hatai found 69.29 per cent (Table I); see the explanation in the preceding paragraph.

Thus duplicate analyses of the same material yield in the case of the human callosum results which are very nearly alike (Table V). The differences noted above are therefore probably not due to technical errors and must arise from other causes.

It further appears that the extract fractions from the human corpus callosum differ by somewhat less than two points (1.54 per cent) from those for the dog (Table VII).

This difference probably corresponds to a difference in the amount of sheathing material in the several animals from which the samples were taken. On the average the value for the extract fraction in man (Table VII) is a trifle greater than for the dog, and in view of the fact that the lowest value found for man is above the highest value found for the dog this may mean that the sheathing substance of the callosal fibers of man is a trifle more developed, though it would perhaps be safer to conclude that in this character man and dog were nearly alike.

As regards the intradural nerve roots, the agreement is less close. While the extract fraction for the nerve roots in the dog (Table II) is 71.46 per cent or about two points (2.14 per cent) above that for the callosum, in the case of man it is only 64.71 per cent or about seven points (6.47 per cent) *below* that for the callosum.[13] In considering this difference it must be borne in mind that the human material was from subjects dead of presumably non-nervous diseases, while the dogs were killed in health. Still, were disease the cause of the difference we should be obliged to assume, either that the values as found for the callosum in man were also too low or that the pathological conditions had not modified the sheathing substance in the callosum.

Though the pathological conditions may have had some influence in bringing about this result, it seems at the moment more plausible to think of the species difference between the two animals, involving as it does a difference in the relative use of the hind limbs, as possibly the more important condition.

[13] It must be remembered that the samples for the callosum and the samples for the nerve roots were taken from different individuals.

If we should accept the sulfatides (lipoid sulfur) as an index of myelination (Table V, 5, F, G, and Table VI, 5, E, F) we should say that the two forms of white matter here compared are very close. It remains to be determined, however, whether this index holds good in other relations before we can accept it as significant.

CONCLUSIONS.

From this survey we conclude that the quantity of the sheathing substance appears to be nearly the same in the callosum fibers of man and of the dog. Moreover, the amount of sheathing substance of the fibers of the intradural nerve roots is in the dog like that found in the callosum, although a higher percentage of phosphatide and a lower percentage of water-soluble phosphorus appear to be characteristic for the nerve roots in both man and the dog. In man, however, the intradural nerve roots appear to have somewhat less sheathing substance, possibly a species difference, possibly due to some other cause.

The approximate similarity in the amount of sheathing substance formed on the peripheral and on the central nerve fibers (though the latter are devoid of neurilemma) in two of the higher mammals, and the fact that the amount of sheathing substance in the callosum of man is similar to that in the callosum of the dog—and in the case of the intradural nerve roots not so very different—are the large results to which attention is especially directed.

THE EFFECT OF DIFFERENT SALTS ON AMMONIA FORMATION IN SOIL.*

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(Received for publication, June 6, 1917.)

It is generally known and has recently been proven by Tottingham and Shive with plants in nutrient solutions of controlled concentrations that the high concentrations of salts are injurious to plants, while the same combination of salts at lower concentrations did not retard their growth. Likewise the writer, controlling the concentration of the solution, studied the effects of a three salt solution (the physiological balance in nutrient solution) upon the decomposition of dialyzed peptone by a pure culture of bacteria and found the same effects. Consequently in order to rule out osmotic differences all solutions used in the work here presented were made up to an osmotic pressure of two atmospheres.

In making up the solutions from the three salts, the triangular diagram known as the Gibbs method, which was used satisfactorily by Schreiner and Skinner and by Shive, was used. An addition to this method was made, however; solutions representing combinations of salts outside the triangle were considered.

To 100 gm. quantities of a "sassafras" soil with which were mixed 155 mg. of nitrogen in the form of dried blood, different combinations of the three salts, $MgSO_4$, K_2SO_4, and $Ca(H_2PO_4)_2.2H_2O$, which were used in a previous work by the writer were applied. The mixture was incubated for a period of 8 days and the ammonia distilled off by the usual method. The determinations were made in duplicate or triplicate and in the most important solutions four determinations were made.

The total concentration of all the solutions employed was two atmospheres. Tottingham clearly discussed the method of calculating the partial osmotic concentration and molecular concentration of a series of salts in combination in order to secure a certain total concentration. The same

* This paper was prepared to be presented at the Biological Chemical Section of the American Chemical Society at its session in Kansas City, Missouri, April 10, 1917.

method was employed throughout this work. It was assumed that the degree of ionization of the salts in every combination was the same as if each were used separately.

No doubt the effect upon the total concentration of the solution when this was in the soil, due to the differential absorption of the salts, was considerable, as shown by Bouyoucos and McCool, but this matter was not brought into consideration at this time.

The following data show the effect of various combinations of a three salt solution upon the composition of dried blood in a soil when the total concentration of the salts applied is two atmospheres.

Molecular proportions of salts.			Ammonia produced.
$MgSO_4$	K_2SO_4	$Ca(H_2PO_4)_2.2H_2O$	
1	1	8	126.6
1	0	9	126.8
0	1	9	126.4
0	0	10	126.7
1	8	1	107.6
0	9	1	107.7
1	9	0	90.5
0	10	0	88.0
8	1	1	106.0
9	0	1	101.0
9	1	0	87.0
10	0	0	85.6
1	4	5	122.6
0	5	5	120.7
4	5	1	104.5
5	5	0	85.0
5	0	5	124.7
4	1	5	120.5
No treatment....................................			100.0* (64.0)

* The amount of decomposition in the soil receiving no treatment of salts was always taken as 100.0 per cent and the decomposition in the others is expressed in terms of this. The actual amount of decomposition in mg. of N of the "no treatment" is given in parentheses.

SUMMARY.

Utilizing various combinations of $MgSO_4$, K_2SO_4, and Ca-$(H_2PO_4)_2.2H_2O$, and controlling the concentration at two atmospheres, the following effects on ammonia formation from dried blood in soil were obtained.

(*a*) In combinations of the salts where $Ca(H_2PO_4)_2.2H_2O$ was present in only 0.1 of the total concentration a considerable increase in ammonia formation was apparent.

(*b*) When 0.8, 0.9, or all of the total concentration was supplied by $Ca(H_2PO_4)_2.2H_2O$ the ammonia formation was approximately 26.0 per cent greater than when no salts were added to the soil.

(*c*) $MgSO_4$ and K_2SO_4 singly or in combination were toxic where no $Ca(H_2PO_4)_2.2H_2O$ was added in the combination.

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THE BEHAVIOR OF CHICKENS RESTRICTED TO THE WHEAT OR MAIZE KERNEL.* II.

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Plate 3.

(Received for publication, July 9, 1917.)

The popular view prevails that among the cereal grains wheat is of superior nutritive worth. Our numerous experiments with mammals[1] point to the contrary view and leave little room for doubt that the wheat grain contains a mildly toxic material. In addition, its proteins are of inferior quality and in a measure may be responsible for some of the malnutrition we have observed where wheat was fed excessively. The fact, however, that the corn kernel proteins are equally inferior for growth, but that this grain is not otherwise deleterious to normal nutrition would most probably place the responsibility for lower nutritive value of the wheat kernel upon some inherent toxic substance or substances.

With chickens, started at half their mature normal weight, we found[2] that they could make slow growth, maintain themselves, and produce fertile eggs on rations limited to corn meal, gluten feed, and calcium carbonate, or wheat meal, wheat gluten, and calcium carbonate. These results are in marked contrast to our records with swine where either of the above rations leads eventually to loss of weight, cessation of oestrum, and in the case of wheat to a condition resembling polyneuritis.

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[1] Hart, E. B., McCollum, E. V., Steenbock, H., and Humphrey, G. C., *Wisconsin Agric. Exp. Station, Research Bull. 17*, 1911; *J. Biol. Chem.*, 1912–17.

[2] Hart, E. B., Halpin, J. G., and McCollum, E. V., *J. Biol. Chem.*, 1917, xxix, 57.

The tolerance of wheat, without ill effects, and the growth and maintenance made by half grown or mature chickens when limited to this grain emphasize the difference in behavior of species in respect to resistance and nutritional requirements.

These early observations, however, left unsettled the question as to the effect of excessive wheat feeding on younger chickens and for that reason these studies were continued. In the experiments to be reported in this paper birds were chosen weighing from 2 to 3 pounds, thus giving opportunity for the animal to make considerable growth. The records secured are not in harmony with those made with nearly grown or mature chickens. The younger birds tolerate the wheat very much less effectively than do those more mature and then only when the mineral content of the ration is adjusted, the proteins of the wheat improved by the addition of casein, and a more liberal supply of the fat-soluble vitamine (from butter fat) added to the ration. Adjustment of a single nutritive factor in the case of feeding wheat to partly grown chickens was insufficient for normal nutrition, although it is evident from our records with the corn grain that the mineral requirements of the growing chicken are essentially different from those of the growing mammal. We have records (to be published later) where baby chicks have grown quite as well on the corn grain fortified only with casein and common salt as on corn grain fortified with casein, common salt, and a complex salt mixture.

On a monotonous diet the mineral content of the ration, to be physiologically balanced, must possess a rather definite qualitative and quantitative composition. Undoubtedly there may exist some latitude in this composition; but one of the most important functions of the salt mixture of a diet is to keep the epithelial cells of the digestive tract functioning normally. This is quite as important as meeting the "needs" of construction and we recognize it as one of the important problems in a study of the mineral requirements of animals on restricted and unvarying diets.

EXPERIMENTAL.

For this work vigorous pullets (Rhode Island Reds) were selected. There were three in a lot, confined to wire cages, with shavings as litter and scratch. They were placed in the cages on

October 15 and the experimental observations terminated June 25, a period of about 8 months' observation. Distilled water was used for all lots and quartz grits were placed in the hoppers. The salts were mixed intimately with the feed whether fed as dry or wet mash. Calcium carbonate was used as the precipitated carbonate in the proportion of 3 pounds to 100 pounds of air-dried feed. Other details of experimentation were precisely as described in our earlier publication.[2] The feeds used in the check lots consisted of 3 parts of corn, 2 parts of wheat, 1 part of oats as scratch, and 1 part each of bran, middlings, and corn as mash. These were supplied at the rate of 2 parts of scratch feed to 1 of mash. The records of the rations fed, growth and behavior of birds, etc., are recorded in Table I.

The striking thing to be observed in the table is the fact that at the end of 3 months' time there was a mortality of 100 per cent in the wheat-fed lots, with the single exception of the lot receiving wheat fortified with casein, butter fat, and a salt mixture. Only two birds had been lost in the three corn kernel lots. The butter fat and casein additions appeared to be the most helpful adjuvants for counteracting the depressing action of continued wheat feeding to this species. This statement rests upon the fact that the addition of a complex salt mixture to the corn kernel and its endosperm proteins did not improve it for the growth and maintenance of chickens and the mineral content of the wheat grain is not strikingly different from that of the corn kernel. The birds that died became greatly emaciated, but no other symptoms of striking character developed. However, when excited, they would often be seized with serious spasms, provoking extraordinary flapping of the wings, followed by exhaustion and collapse. This would indicate a derangement in the nervous system. Excessive wheat feeding to cattle or swine ultimately induces pathological changes in the nervous tissue.[3] It is possible that such changes were occurring here, but up to the present time no histological studies have been made.

The corn grain fortified only with its endosperm proteins and calcium carbonate suffices for growth and the continued main-

[3] Hart, E. B., Miller, W. S., and McCollum, E. V., *J. Biol. Chem.*, 1916, xxv, 239. Hart, McCollum, Steenbock, and Humphrey, *J. Agric. Research*, 1917 (in press).

TABLE I.

Record of Results 1916–17.

Duration, October 15, 1916, to June 30, 1917. Laying Period April 16 to June 23.

Ration.	Bird No.	Weight.					Date of death.	Final weight.	Eggs collected.
		Oct. 15	Dec. 15	Jan. 15	Feb. 15	June 15			
		lbs.	*lbs.*	*lbs.*	*lbs.*	*lbs.*		*lbs.*	
Corn meal 70 lbs.	801	3.06	3.43	3.43	3.62	3.5		3.62	0
Gluten feed 30 "	802	2.25	2.00	Dead.			Dec. 1916	1.50	0
$CaCO_3$ 3 "	803	2.56	3.50	3.75	4.31	3.31		3.68	8
Quartz.									
Corn meal 70 lbs.	804	2.93	3.31	3.18	3.37	2.63		2.75	10
Gluten feed 30 "	805	2.18	2.00	1.68	1.75	Dead.	Apr. 1916	1.37	0
$CaCO_3$ 3 "	806	2.16	3.18	3.16	3.37	3.43		3.43	3
K_2HPO_4 323 gm.									
Ca lactate 513 "									
Quartz.									
Corn meal 95.5 lbs.	819	3.06	4.5	4.62	5.00	4.12		4.37	
Casein 2.5 "	820	2.81	3.87	3.75	3.81	3.56		3.56	0
K_2HPO_4 323 gm.	821	2.06	2.37	2.25	2.43	2.75		3.30	6
Ca lactate 513 "									0
$CaCO_3$ 3 lbs.									
Quartz.									
Wheat meal 95.5 lbs.	807	2.68	2.5	Dead.			Dec. 1916	1.75	0
Wheat gluten 2.5 "	808	2.62	1.75	"			" 1916	1.75	0
$CaCO_3$ 3 "	809	2.25	3.31	3.00	Dead.		Jan. 1917	3.00	0
Quartz.									
Wheat meal 95.5 lbs.	810	3.25	2.87	Dead.			Jan. 1917	1.81	0
Wheat gluten 2.5 "	811	2.31	1.93	"			Dec. 1916	1.25	0
K_2HPO_4 323 gm.	812	2.31	1.75	"			" 1916	1.50	0
Ca lactate 513 "									
$CaCO_3$ 3 lbs.									
Quartz.									
Wheat meal 95.5 lbs.	813	2.75	2.25	Dead.			Dec. 1916	2.00	0
Casein 2.5 "	814	2.56	2.50	"			" 1916	2.00	0
K_2HPO_4 323 gm.	815	2.12	2.12	"			Jan. 1917	1.25	0
Ca lactate 513 "									
$CaCO_3$ 3 lbs.									
Quartz.									

TABLE I—*Concluded.*

Ration.	Bird No.	Weight.					Date of death.	Final weight.	Eggs collected.
		Oct. 15	Dec. 15	Jan. 15	Feb. 15	June 15			
		lbs.	*lbs.*	*lbs.*	*lbs.*	*lbs.*		*lbs.*	
Wheat meal 95.5 lbs.	816	3.12	3.87	3.00	2.25	Dead.	Feb. 1917	2.25	0
Casein 2.5 "	817	2.56	4.06	4.68	5.25	4.37		4.50	6
Butter fat 2 "	818	1.87	2.50	1.75	Dead.		Jan. 1917	1.75	0
K_2HPO_4 323 gm.									
Ca lactate 513 "									
$CaCO_3$ 3 lbs.									
Quartz.									
Check, variety grain ration.	822	2.75	3.37	3.00	3.18	3.5		3.00	6
	823	2.00	Dead.				Nov. 1916	1.12	0
$CaCO_3$ 3 lbs.	824	2.50	4.00	4.25	4.37	3.43		3.50	16
Quartz.									
Milk.									
Check, variety grain ration.	825	3.00	4.31	4.00	4.75	3.75		4.25	4
	826	2.93	2.87	2.75	2.87	Dead.	Feb. 1917	2.50	0
$CaCO_3$ 3 lbs.	827	1.81	2.62	2.25	Dead.		" 1917	2.00	0
Quartz.									

tenance of this species. From these data and those published earlier[2] it is apparent that the corn kernel can supply adequately the vitamine demands (both fat-soluble and water-soluble) of growing and reproducing chickens.

Figs. 1 to 3 contrast the condition of certain of these animals after 75 days on the ration. Even after 8 months' use of these rations exactly similar conditions prevailed; the birds fed the corn grain, gluten feed, calcium carbonate ration continued to thrive, as also did one from the group of chickens fed the wheat, casein, and butter fat, and a salt mixture.

SUMMARY.

1. Chickens started below their half normal weight can make slow growth and maintain themselves on rations restricted to corn meal, gluten feed, and calcium carbonate. But a ration restricted to the wheat grain, wheat gluten, and calcium carbonate will terminate life in this species in 3 months. These re-

sults harmonize with our data relative to the mammal, particularly in respect to the action of the wheat grain.

2. These and our earlier records illustrate, however, a difference in the behavior of more mature and younger chickens. The former will tolerate wheat, but the latter, like either mature or young mammals, will not tolerate excessive wheat feeding. There is also a difference in the mineral needs of growing chickens and growing mammals.

3. Modifying the wheat grain by the addition of a complex salt mixture as a single change, or including an additional alteration, such as substituting casein for part of the wheat protein, did not appear to improve the dietary properties of this grain for young growing chickens. Only when butter fat was added and the casein-mineral modification also included was there complete tolerance and well-being.

4. These results do not mean that wheat or wheat products cannot be fed growing chickens or mammals, but only point out their dietary limitations with another species. The data also show that the wheat grain cannot be used exclusively and successfully for young growing chickens without the introduction of other dietary factors, and indicate what those factors are.

EXPLANATION OF PLATE 3.

Fig. 1. Condition of a fowl at the end of 3 months' on a ration of 70 pounds corn meal, 30 pounds gluten feed, 3 pounds $CaCO_3$, distilled water, and quartz. From all appearances this was a normal bird.

Fig. 2. After 3 months on a ration of 95.5 pounds wheat meal, 2.5 pounds wheat gluten, 3 pounds $CaCO_3$, distilled water, and quartz. Dead when photographed. Somewhat emaciated. A typical condition of exclusively wheat-fed birds without complete modification of the ration.

Fig. 3. After 3 months on a ration consisting of 95.5 pounds wheat meal, 2.5 pounds casein, 2 pounds butter fat, 3 pounds $CaCO_3$, 513 gm. calcium lactate, 323 gm. K_2HPO_4, quartz, and distilled water. Apparently normal condition, although this was the only bird in the four wheat-fed lots that survived and then only when the wheat meal, wheat gluten mixture was modified in respect to proteins, ash, and the amount of the fat-soluble vitamine.

Fig. 1

Fig. 2.

Fig. 3.

(Hart, Halpin, and Steenbock: Wheat or Maize Rations.)

STUDIES IN CALCIUM AND MAGNESIUM METABOLISM.

I. THE EFFECTS OF BASE AND ACID.*

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Physiological literature contains comparatively few statistics dealing with the effect of acids and bases on the metabolism of calcium and magnesium.[1] In their well known compilation on mineral metabolism Albu and Neuberg state:

> The amount (of calcium) absorbed depends in part upon what other salts are taken with the nourishment, especially does sodium chloride increase the absorption of calcium and on the contrary the addition of alkalies diminishes it.

No figures or experiments are given in support of this statement. The present investigation is primarily the outcome of a consideration of the claim of Dubois and Stolte that storage of calcium is dependent upon a suitable supply of alkali to the organism. By the addition of alkali carbonates to the food of children these investigators found that they could change a negative calcium balance to a positive one. They believed this outcome to be due to the neutralization, by the alkali, of the phosphoric and sulfuric acids formed in metabolism; also to a prevention of the formation in the alimentary canal of insoluble calcium soaps which cannot be utilized. According to the first consideration, by supplying alkali the unnecessary withdrawal of calcium as a

* The data in this and the two papers following are taken from the dissertation presented by Maurice H. Givens for the degree of Doctor of Philosophy, Yale University, 1917.

[1] On administration of acid see Heiss, Gaehtgens, Rüedel, Caspari, Granström, and Secchi; on administration of bases see Dubois and Stolte, Bertram, Magnus-Levy, and Gerhardt and Schlesinger.

neutralizing agent for acids formed in metabolism is averted; and in the second instance the loss of unabsorbed calcium by the bowel is prevented.

Methods.

The general plan of the present studies involves metabolism experiments in which the income and outgo and corresponding balance of various elements were ascertained under diverse conditions of diet. In preliminary experiments planned to test the available methods for the estimation of calcium it was found that because of the small amount of calcium present in dog urine, the precipitation of calcium as the oxalate, by McCrudden's method, resulted in a precipitate too fine to be filtered quantitatively. The difficulty was overcome by concentrating the urine. The acid urine was evaporated on the water bath to a small volume and then precipitated as directed by McCrudden, particular attention being paid to the reaction. The precipitate was allowed to stand over night, filtered on a blue ribbon S and S paper, washed two or three times with a 0.5 per cent solution of ammonium oxalate, dried, burned, dissolved in hydrochloric acid, filtered, and reprecipitated according to McCrudden's procedure for human urines. All filtrates were combined for magnesium estimation and treated according to the original description of the McCrudden method. In all cases the precipitate was ignited and weighed as calcium oxide. The latter was frequently dissolved in standard hydrochloric acid solution and titrated with standard sodium hydroxide solution to show that the precipitate was uncontaminated.

The calcium and magnesium in the ash of foods and feces were determined by McCrudden's procedure.

Phosphorus was estimated in urine by titration with uranium solution, and in the ash of feces and dried milk by the gravimetric molybdic method.

Nitrogen was determined in all cases by a Kjeldahl method.

The meats fed were bought in large quantities, cleaned free from visible fat and fascia, hashed, weighed out in 250 gm. packages, wrapped in paraffined paper, frozen, and kept in storage at 30° F. or lower. All meats kept perfectly under these conditions.

For the first set of experiments thoroughly dried powdered bread was used. Later cracker meal was substituted.

The dried skimmed milk was Merrell-Soule Company's "Klim."

Dogs B and F were catheterized every 48 hours with due antiseptic precautions. There was never a sign of cystitis. From the other dogs the spontaneously voided urine was collected at definite intervals.

Feces were marked off by feeding approximately 0.5 gm. of ground cork.

TABLE I.

Analyses of the Components of the Diets.

	Water.	N	Fat.	Ash.	CaO	MgO	P
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Meat H_1	58	2.57	19.5	0.63	0.035	0.021	
" 2	72	3.55	(2.8)	1.03	0.011	0.033	
" 3	74	3.54	(2.8)	1.04	0.009	0.037	
" 4	74	3.84	(2.8)	1.10	0.008	0.036	
" H_2	63	2.77	15	0.75	0.032	0.020	
" 5	73	3.38	(2.8)	1.03	0.011	0.037	
Dried bread		2.15	0.5	1.95	0.092	0.047	
Cracker meal 1	(9.0)	1.76	(6.0)	2.43	0.038	0.031	
" " 2	(9.0)	1.66	(6.0)	2.30	0.036	0.034	
Dried skimmed milk (Klim)		5.66		8.01	1.703	0.384	0.85
Agar-agar				11.93	1.03	0.41	

New Haven tap water of October 9, 1916, contained SiO_2 0.006, CaO 0.024, and MgO 0.004 gm. per liter.

The values in parentheses are calculated from the tables of Atwater and Bryant.

Composition of Daily Diets.

Diet.	N	Fat.	Carbohydrate.	CaO	MgO	P*	Estimated calories.**
	gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	
A	8.57	48	78	186	100	617	994
B	10.10	51	51	223	170	602	952
B1	10.10	47	60	405	207	710	952
B2	10.08	51	51	160	155		952
B3	10.01	51	51	158	158		952
B4	9.61	51	51	162	158		952
B5	10.43	50	36	148	149		872
A2	7.50	38	25	200	103		648

* All phosphorus figures are taken from Sherman and Gettler except those of the dried skimmed milk.

** Calories estimated from the tables of Atwater and Bryant.

EXPERIMENTAL.

If alkali or acid influences either the nitrogen, calcium, magnesium, or phosphorus metabolism it should be revealed by adding these substances to calcium-poor and calcium-rich diets. Accordingly such experiments were carried out on two bitches.

Dog B weighed 13.2 kg. at the beginning of the experiment (October 16, 1916) and 14 at the end (March 2, 1917). Dog F weighed 14.6 kg. at the beginning of the experiment (November 21, 1916) and 16.6 at the end (March 2, 1917). Table I gives the composition of the food intakes. Agar was added to the food mixture to produce a daily evacuation of the bowel in order to lessen the possibility of reabsorption of lime. It also served the purpose of producing well formed stools. There was no diarrhea.

Unfortunately the addition of agar had been going on for some time before it was realized that this substance contains considerable calcium (about 1 per cent). The water intake was limited. The amount of sodium bicarbonate given was approximately 0.5 gm. per kilo. The exact amount fed is indicated in Tables II and III, likewise the exact amount of hydrochloric acid, introduced into the stomach by sound. The average daily results are included in Tables II and III.

Balance of Nitrogen.

In the case of Dog B there was a positive *nitrogen* balance for all periods except the last three. The final negative nitrogen balance may be due to the fact that in Period 20 the dog received 5.1 gm. of calcium lactate daily for 6 days. This may have upset the animal as the usual dose of calcium lactate for man is 0.5 gm.,[2] while a dose of 20 gm. daily will produce untoward symptoms.[3] It may be that the dog's negative nitrogen balance was due to the fact that she had been kept in the cage for such a long time.

The experiments on Dog B with dried skimmed milk, alkali, and calcium lactate were repeated on Dog F (Table III). The

[2] Sollman, T., A Manual of Pharmacology, Philadelphia, 1917.

[3] Towles, C., *Am. J. Med. Sc.*, 1910, cxl, 100.

TABLE II.

Nitrogen, Calcium, Magnesium, and Phosphorus Metabolism, Averages per Day. Dog B.

Period.	Intake.		Urine.				Feces.				Balance.			
	Standard diet.	Daily additions.	N	Ca	Mg	P	N	Ca	Mg	P	N	Ca	Mg	P
			gm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*	*mg.*	*mg.*	*mg.*
1	A		6.87	15	28	370	680	315	45	86	+1.01	−196	−14	+160
2	A	6.5 gm. $NaHCO_3$.	6.55	11	28	356	357	115	41	43	+1.66	+9	−8	+220
3	B		7.76	9	33	458	256	85	37	35	+2.09	+26	+31	+110
4	B	6.5 gm. $NaHCO_3$.	8.38	11	34	480	607	179	70	74	+1.11	−73	−18	+50
5	B		8.26	9	31	464	570	186	59	76	+1.27	−80	−3	+60
6	B_1	0.34 gm. CaO in milk.	8.36	9	25	450	455	288	65	116	+1.25	+64	+34	+110
7	B		8.39	11	39	476	633	172	69	66	+1.08	−64	−21	+60
8	B_1	0.34 gm. CaO in milk + 6.5 gm. $NaHCO_3$.	9.06	9	35	320	491	322	65	143	+0.55	+36	+24	+250
9	B		8.99	9	36	336	542	150	75	46	+0.56	−36	−23	+220
10	B	About 1.5 gm. HCl.	8.56	25	45	460	432	136	70	51	+1.10	−50	−28	+90
11	B		8.39	9	31	216	592	178	90	80	+1.12	−71	−4	+300
12	B_1	0.34 gm. CaO in milk + 1.5 gm. HCl.	8.88	19	28	240	485	293	109	160	+0.73	+43	−12	+310
13	B		9.39	9	29	215	—	—	—					
14	B_2		8.91	10	27		487	150	49		+0.68	−43	+17	
15	B_2	6.5 gm. $NaHCO_3$ 0.1–0.3 gm. CaO as Ca lactate.	9.04	17	38		547	288	69		+0.50	−43	−15	
16	B_{2+3}	6.5 gm. $NaHCO_3$ 0.4–0.6 gm. CaO as Ca lactate.	9.08	19	31		547	485	54		+0.44	−36	+9	
17	B_3	6.5 gm. $NaHCO_3$ 0.7 gm. CaO as Ca lactate.	8.84	17	24		495	558	74		+0.67	+40	−6	
18	B_3	"	8.96	22	32		350	565			+0.70	+21		
19	B_{3+4}	0.7 gm. CaO as Ca lactate.	9.30	14	34		461	615			+0.08	−21		
20	B_4	1.0 gm. CaO as Ca lactate.	10.16	15	37		481	630			−1.03	+185		
21	B_4	6.5 gm. $NaHCO_3$ 1.0 gm. CaO as Ca lactate.	9.81	22	24		565	728			−0.77	+71		
22	B_4		10.07	12	36		400	140			−0.86	−43		

TABLE III.

Nitrogen, Calcium, Magnesium, and Phosphorus Metabolism, Averages per Day. Dog F.

Period.	Intake.		Urine.				Feces.				Balance.			
	Standard diet.	Daily additions.	N	Ca	Mg	P	N	Ca	Mg	P	N	Ca	Mg	P
			gm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*	*mg.*	*mg.*	*mg.*
1	B		8.26	35	25	326	450	200	68	70	+1.39	−117	−6	+225
2	B	7.25 gm. $NaHCO_3$.	8.43	33	26	219	740	300	97	74	+0.43	−217	−36	+330
3	B		8.81	33	28	182	680	272	80	103	+0.61	−190	−21	+330
4	B_1	0.34 gm. CaO in milk.	9.62	31	28	185	800	573	125	223	−0.32	−243	−28	+330
5	B		9.51	37	30	210	630	272	81	109	−0.04	−196	−24	+300
6	B_1	7.25 gm. $NaHCO_3$ 0.34 gm. CaO in milk.	9.43	31	29	192	670	450	87	188	−0.002	−123	+8	+450
8	B_2		8.54	30	19		660	258	45		+0.88	−173	+29	
9	B_2	0.1–0.3 gm. CaO as Ca lactate.	8.32	31	21		760	415	82		+1.00	−186	−10	
10	B_2	0.4–0.6 gm. CaO as Ca lactate.	8.36	32	19		690	586	80		+1.00	−143	−4	
11	B_3	0.7 gm. CaO as Ca lactate.	8.36	33	15		630	658	71		+0.97	−79	+9	
12	B_3	"	8.52	34	16		710	708			+0.77	−129		
13	B_{3+4}	0.7 gm. CaO as Ca lactate. 6.5 gm. $NaHCO_3$.	8.63	32	17		890	790			+0.36	−214		
14	B_4	1.0 gm. CaO as Ca lactate. 6.5 gm. $NaHCO_3$.	8.66	33	18		740	885			+0.21	−93		
15	B_4	1.0 gm. CaO as Ca lactate.	8.59	32	17		790	850			+0.26	−57		
16	B_4		8.84	27	22		640	220			+0.13	−7		

basal meat-cracker meal diet was essentially the same as that of the preceding series. The *nitrogen* balance was favorable throughout except in Periods 4, 5, and 6 when the animal, unaccustomed to the cage, developed a slight disability which was promptly relieved by a few days transfer to a large pen. As evidence of good health the continued gains in body weight over a period of 101 days may be cited.

Balance of Calcium.

On the basal meat-cracker meal or calcium-poor diet Dog B was constantly in negative calcium balance with one exception. This was near the beginning of the experiment (Period 3) and probably before the dog was thoroughly drained of its reserve (?) of lime.

After the addition of sodium bicarbonate to the basal diet A one would have expected an approach to a positive calcium balance, if the argument of Dubois and Stolte were tenable. In only one instance (Table II, Period 2) did this occur; and then we believe it was due to the meat used. (In Periods 1 and 2 there was used with Dog B a Hamburg hash purchased in the open market. Such a meat is liable to have bone ground in with it. This conclusion is confirmed if one refers to the calcium content of hashes H_1 and H_2 in Table I and then compares them with the calcium content of the other meats which were more carefully prepared under our direction. No bones were sawed in these latter meats and all tendons and fat were carefully removed.) We cannot consider this slight positive balance of 55 mg. of calcium oxide in 6 days of especial significance, further than to say that the dog was in calcium equilibrium. The methods of marking off the feces are still inevitably too crude to permit of nice discriminations involving a few mg. of calcium out of a total of several hundred eliminated per day.

When the experiment with sodium bicarbonate was repeated (Table II, Period 4) the dog showed a distinct negative calcium balance daily amounting to almost half of the fecal excretion of lime.

Inasmuch as the alkali had no effect on the calcium utilization of the meat-cracker meal diet the effect of hydrochloric acid in-

troduced by sound into the stomach was next tried. The dog was fed as usual and in 1 to 1½ hours approximately 1.5 gm. of hydrochloric acid were introduced. The dog retained the acid without any apparent untoward signs. The acid produced no effect on the balance of calcium which was negative in about the same magnitude as in the preceding and following periods. The acid did, however, have the striking effect of increasing the urinary calcium about 200 per cent (Table II, Period 10) presumably diverting some of the lime from the intestinal path.

In view of the essentially negative influence of base and acid on calcium metabolism, during a calcium-poor diet, it seemed desirable to repeat the experiment during an increased dietary calcium intake. Accordingly dried skimmed milk was substituted in nitrogen equivalent for the cracker meal, and the caloric intake was maintained constant by the addition of sucrose to the food. In this way the lime intake of the preceding diet was more than doubled.

When 0.34 gm. of calcium oxide, contained in 21.7 gm. of dried skimmed milk, was added to the diet, the negative calcium balance of the meat-cracker meal régime was turned into a distinctly positive one. When further 6.5 gm. of sodium bicarbonate were daily added to the milk ration, the positive calcium balance decreased one-half. Exactly the same results as on the milk plus alkali were obtained by introducing into the dog's stomach 1.5 gm. of hydrochloric acid. To recapitulate: the milk changed a negative to a positive lime balance; this balance was not increased by either acid or alkali, but conversely, reduced about half by both.

The addition of the lime in the milk period produced no effect on the calcium content of the urine, but of course increased the fecal output. As on the basal diet, the addition of the acid to the food nearly doubled the urinary calcium. Relatively, the increase in the lime of the urine was large, but absolutely it was small (15 to 23 mg. per day).

Following this lead the basal calcium-poor diet was next supplemented with a soluble calcium salt—calcium lactate (Merck's soluble). The initial dose of 510 mg. of air-dry calcium lactate on analysis yielded 100 mg. of calcium oxide. This was increased every other day by 510 mg. until the dog was receiving 3.57 gm.

of calcium lactate per day for 18 days and then 5.10 gm. daily for 12 days. Except in Periods 14, 20, and 22 the dog also received daily 6.5 gm. of sodium bicarbonate. Carbonates were always present in the urine during the periods of alkali administration.

An inspection of the results recorded in Table II shows that between the limits of 0.51 and 3.06 gm. of calcium lactate intake per day, with the addition of 6.5 gm. of sodium bicarbonate daily there remained a negative calcium balance of about the same magnitude as in Period 14 on the basal diet alone. Only when the daily calcium intake was raised to 3.53 gm. of calcium lactate plus the sodium bicarbonate was there a slight positive calcium balance.

It might appear from the results in Period 19, where a slight negative calcium balance occurred in the absence of alkali administration despite a daily intake of 3.53 gm. of calcium lactate, that the alkali actually facilitated the storage of lime. This interpretation is unlikely, however, since on the higher intake of calcium in Periods 20 and 21 no advantage whatever appeared in respect to calcium balance when alkali was administered. In fact the calcium balance on lime alone (Period 20) was more favorable than when alkali was furnished (Period 21).

A survey of Table II supports the conclusion that the addition of sodium bicarbonate has not had any marked influence upon the calcium metabolism. The striking fact is that 0.34 gm. of calcium oxide per day in 21.7 gm. of dried skimmed milk, regardless of base or acid, will produce a positive calcium balance; whereas 1 gm. of calcium oxide in the form of calcium lactate is necessary to accomplish the same end. The problem of the relative advantage of the combination in which calcium is fed is thus raised anew.

In the case of Dog F it is interesting to note that, for a period of 52 days, the dog had a positive nitrogen balance of 31.6 gm. and a negative calcium oxide balance of 8.7 gm., even though she was receiving extra lime on all except 10 days. In this animal the addition of dried milk or calcium lactate, each alone or with alkali, failed to convert the negative calcium balance of the basal diet into a positive one. *Here again the alleged favorable influence of alkali upon calcium storage was missed.*

Balance of Magnesium.

The magnesium balance of Dog B on the first basal diet A was negative and was not changed by the addition of alkali. When the basal diet B was instituted there was a change to a temporary positive magnesium balance due presumably to the increased intake of magnesium in the meat. Subsequently it became negative until milk was introduced into the food. The alkali did not essentially alter the magnesium balance with any of the diets used.

When hydrochloric acid was superimposed upon the dried skimmed milk diet the magnesium balance became negative. The positive daily magnesium balance upon the skimmed milk either without or with alkali is greater than the daily increment of magnesium in the milk. This suggests determinative factors other than the magnesium *per se*. The same features appear in the record of Dog F.

The variability of the magnesium excretion, when calcium salts are added to the diets, was such that one cannot draw a conclusion as to whether the increased intake of lime altered the excretion of magnesium or not.

Balance of Phosphorus.

The phosphorus balance was always positive though extremely variable quantitatively. The figures for both the urinary and fecal phosphorus were so variable that neither could be used as an index of any influence exerted by sodium bicarbonate administration. The increased phosphorus intake derived from the milk was eliminated in part by the kidneys and in part by the intestines.

One would expect on the basis of available information[4] that more phosphorus would be eliminated through the intestines when alkali is included in the diet and through the kidneys when acid is administered. In general this has proved to be the case in the present experiments.

[4] For a review of the literature of phosphorus compounds in animal metabolism see the compilation of Forbes, E. B., and Keith, M. H., *Ohio Agric. Exp. Station Technical Bull. 5*, 1914.

The Relation of Calcium to Magnesium.

The relation of calcium to magnesium has been studied by several investigators. The antagonistic action of calcium to magnesium anesthesia has been clearly shown by Meltzer and his collaborators.

Malcolm has presented considerable evidence to show that the ingestion of soluble magnesium salts causes a loss of calcium in adult dogs and hinders its deposition in young growing rats, while soluble calcium salts do not in the same way promote the excretion of magnesium.

Mendel and Benedict observed that the injection of calcium salts was followed by an increased elimination of magnesium in the urine; similarly the injection of a magnesium salt increased the output of calcium.

Tereg and Arnold found, after feeding acid calcium phosphate to dogs, a slightly increased amount of urinary calcium.

Ruedel observed an increasing urinary output of calcium in rabbits and dogs after the injection subcutaneously of calcium acetate.

Hart and Steenbock found that the addition of magnesium salts to the feed of a pig increased its urinary calcium output. The fecal calcium excretion was not influenced.

In our experiments the relation of calcium to magnesium in the urine in the case of Dog B was quite variable; for example, on the basal diet the ratio was 1:2, 1:3, and 1:4. When alkali was added to the basal diet, the Ca:Mg relation was still 1:2.5 and 1:3. The addition of dried skimmed milk did not alter this. The highest output of magnesium was during the dried skimmed milk plus alkali régime, Ca:Mg = 1:4. When acid was superimposed upon the basal diet and the basal plus dried skimmed milk the relation of calcium to magnesium was about 1:1.5 in both cases. This was due to an increased excretion of lime induced by the acid. The magnesium values were too variable to permit of drawing conclusive inferences.

The relations for Dog F were more consistent. The Ca:Mg ratio, in the majority of cases was about 1:1. This relation was not disturbed by the addition of dried skimmed milk to the diet or by the addition of alkali to either the basal diet or the basal diet plus milk. When calcium lactate or alkali and calcium lactate were added to the diet the ratio was changed to 1.5:1; that is to say, under these conditions more calcium than magnesium was excreted through the kidneys.

The Urinary Excretion of Calcium and Magnesium in Human Diabetes during Sodium Bicarbonate Ingestion.

An opportunity was offered to study the urinary calcium and magnesium output of a male diabetic[5] who received 40 gm. of sodium bicarbonate per day. The analytical data and the daily diet are included in Table IV. The urine of the subject always contained an excess of carbonates. If the alkali had had a significantly depressing effect upon the urinary output of calcium, the amount excreted would certainly have been less than here reported.

TABLE IV.

Analyses of the Daily Urinary Excretion of a Diabetic Man. Initial Weight 125 Pounds.

Date.	Volume.	Reaction to litmus.	Total N.	CaO	MgO	P
Nov.	*cc.*		*gm.*	*gm.*	*gm.*	*gm.*
16	3,300	Alkaline.	18.8	0.76	0.16	1.34
17	3,000	"	20.0	0.72	0.18	1.48
19	2,950	"	19.3	0.74	0.14	1.48
20	3,500	"	22.8	0.72	0.16	1.56
21	3,400	"	23.6	0.75	0.18	1.44
Average............	3,230		20.9	0.74	0.16	1.46

During the above period the patient received daily 40 gm. of $NaHCO_3$ and the average daily food consumption was as follows.

	gm.
Casoid flour................	55.5
Bran (washed)............	15–30
Eggs.....................	95
Cooked meat, beef, ham, fish.....................	180–200
Lettuce..................	30
Celery (thrice boiled)......	15–30
Cauliflower (thrice boiled)...	150–180
Butter....................	75–90

	cc.
Olive oil......................	8
Vinegar.......................	3
Beef extract..................	2–3
	gm.
Oleomargarine (in casoid biscuit)......................	18
Lard (in casoid biscuit)........	12

Nelson and Burns found the normal urinary calcium oxide excretion in man to range between 0.18 and 0.62 gm. per day. *In our diabetic the alkali seems not to have influenced the urinary calcium output.*

[5] This case is included in the paper of Underhill, F. P., *J. Am. Med. Assn.*, 1917, lxviii, 497.

That the balance of calcium in the diabetic ordinarily is negative seems to be established from the recent work of Kahn and Kahn who made careful analyses of the intake and output of five diabetics. The literature of the subject is given in their paper.

SUMMARY.

Administration of base or acid produced no significant effect upon the balance of nitrogen, calcium, magnesium, and phosphorus in the dog.

Administration of hydrochloric acid increased the urinary excretion of calcium and thereby altered the relation of calcium to magnesium in the urine.

The calcium contained in milk was more effective than soluble calcium lactate in producing calcium retention.

Administration of large doses of alkali bicarbonate to a human diabetic did not decrease the urinary output of calcium.

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STUDIES IN CALCIUM AND MAGNESIUM METABOLISM.

II. THE EFFECT OF DIETS POOR IN CALCIUM.

By MAURICE H. GIVENS.

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(Received for publication, June 20, 1917.)

Albu and Neuberg have stated that the kind and intensity of calcium exchange is extraordinarily dependent upon the quality of the nourishment.

Forster fed a dog washed meat and showed by analysis of blood and tissues that the dog had lost calcium. The loss from the tissues did not equal the excess of output over intake, therefore the bones must have yielded some calcium.

Perl found a normal urinary calcium excretion in the dog on a calcium-poor diet of bread and a little condensed milk. The output was increased on a heavy meat-lard diet.

Goitein found that rabbits on a calcium-rich diet of oats and bone meal put on calcium and magnesium; with a diet less rich in calcium, as oats alone, the animals were in calcium and magnesium balance; and with a calcium-poor diet, as maize alone, the rabbits might be in nitrogen and magnesium balance but negative calcium balance.

Boekelman and Staal studied the effect of a calcium-poor diet and a calcium-rich diet on calcium excretion in man. The calcium-poor diet was an ordinary mixed ration while the comparison diet had its calcium content a little more than trebled by the addition of a liter of milk. In their four subjects the urinary calcium increased on the calcium-poor diet and decreased on the calcium-rich diet.

Patterson fed rabbits on oat-meal and maize, a diet which, as he says, "admittedly leads to calcium starvation." He found the amount of calcium in the blood in a normal proportion to the rest of the ash; in the bones, however, the relation of the calcium to the total minerals was reduced. He believes: "The bones can, without doubt, act as reservoirs of calcium (and probably of magnesium)."

The most intensive study made of the effect of diet on the calcium and magnesium excretion of dogs is that of Kochmann and his pupil Petzsch. From a study of the effects of protein, fat, and carbohydrate on the alkali-earth metabolism of dogs they concluded that the calcium balance was

apparently influenced by the amount of these organic nutrients, in that an addition of any one of the three substances to the basal ration would draw out lime from the organism in a noticeable degree. They thought the amount of calcium necessary for a maintenance of calcium balance depended upon the character of the food intake and had to be determined for each diet. Unlike the case of calcium metabolism they found magnesium exchange unaffected by protein, fat, and carbohydrate. The phosphorus metabolism likewise is believed to be influenced by the protein intake. These workers did not use a mixed diet. Protein, fat, and carbohydrate respectively were superimposed upon a diet of protein alone. In our experiments we have endeavored to provide a constant caloric intake on a mixed diet.

TABLE V.*

Urinary Alkali-Earth Excretion, Mg. per Kg.

Weight of dog.	CaO	MgO	Diet.	Author.
kg.				
11	0.7	0.8	No food.	Mendel and Benedict.
11	1.5	3.0	" "	Givens.
14	0.5	1.2	Ca-poor.	Mendel and Benedict.
20	1.0	2.5	"	Gaehtgens.
14	1.0	4.0	"	Givens.
20	1.5	1.8	"	Gaehtgens.
15.5	1.8		"	Tereg and Arnold.
16.4	2.0	4.0	"	Kochmann.
10.3	2.2	8.8	"	"
13.5	2.5		"	Ruedel.
24	2.6	5.0	"	Secchi.
13	3.0	3.2	"	Givens.
14	3.0	7.5	"	"
9.3	3.0	7.6	"	Kochmann.
4	3.0	10	"	Heiss.
14.5	3.2	3.2	"	Givens.
12	3.5	4.1	"	"
5.3	5	10	"	Kochmann.
33	1.3		Ca-rich.	Tereg and Arnold.
22	1.5		"	Perl.
7.7	3.4	8	"	Malcolm.
9.6	4	5	"	"
6.5	4.6	12	"	Kochmann.
14	6	7	"	Secchi.
7	16	13	"	Kochmann.

* The tables are numbered consecutively through the three papers.

The results of Kochmann are included in the following résumé (Table V) of all of the available literature on the urinary alkali-earth excretion of *adult* dogs. The statistics fail to show any *definite* relation between the diets and urinary calcium and magnesium. The tendency is for the excretion of these elements to increase as the intake of them increases.

From the literature on the subject we are led to conclude that a diet poor in calcium is not conducive to a storage of either calcium or magnesium despite an abundance of nitrogenous food. This is further established by our own investigations.

The fact that the body may show a satisfactory nutritive balance with respect to one essential element while being depleted of another deserves more emphasis than it has received in the past.[1] There are available for comparison in our series of experiments, the results obtained on five animals; *viz.*, Dogs B,[2] F,[3] J,[4] FL,[5] and M_2 (Table VI). With Dogs B, F, and M_2 there re-

TABLE VI.

Nitrogen, Calcium, and Magnesium Metabolism, Average Daily Analyses. Dog M_2.

Period.	Diet.	Urine.			Feces.			Balance.			Weight.
		N	Ca	Mg	N	Ca	Mg	N	Ca	Mg	
		gm.	*mg.*	*mg.*	*gm.*	*mg.*	*mg.*	*gm.*	*mg.*	*mg.*	*kg.*
1	A_2	5.98	34	49	0.60	270	30	+0.91	−159	−17	13.8
2	"	5.41	23	45	0.72	220	26	+1.30	−99	−11	13.8
3	"	5.52									14.0
4	"	5.46	36	55							14.2
5	"	5.07	33	47							14.3

sulted from the calcium-poor diets a negative balance of calcium and magnesium. The relation of calcium to magnesium in the urine was not altered appreciably in any of the dogs. Whether this would have been the case if the experiments had been con-

[1] Illustration of the significance of this will be found in the compilation of Forbes and others, *Ohio Agric. Exp. Station Bull. 295*, 1916, and *Bull. 308*, 1917.

[2] Table II, p. 425.

[3] Table III, p. 426.

[4] Table VII, p. 442.

[5] Table VIII, p. 442.

tinued over a sufficiently long period cannot be foretold. The excretion of lime in the feces was reduced owing to the decreased intake of the element.

No apparent relation between nitrogen and calcium output is evident in any of the dogs. In almost every instance the nitrogen balance has been positive while the calcium balance has been negative. This might be expected from the nature of the diet, which, though rich in nitrogen was poor in calcium.

The relation between the outputs of calcium and magnesium on the diets here under discussion is by no means constant. Thus with Dog B the urinary magnesium excretion was greater than the calcium. This decided difference may be due to the fact that this old laboratory dog had probably been on a calcium-poor diet for years, receiving no more lime than was actually needed to maintain her health.

The relation of calcium to magnesium in the urine was almost always about 1 : 1 in all of the other animals. Calcium always exceeded magnesium in the feces as 2 : 1 or more in both Dogs B and F. These two animals excreted the most of their phosphorus through the kidneys and its variations did not seem to influence the calcium output.

From the data submitted it is evident that *diets poor in calcium are not conducive to positive calcium* balance even when an abundance of nitrogenous food is available.

My thanks are due Professor Lafayette B. Mendel for his advice and criticism.

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STUDIES IN CALCIUM AND MAGNESIUM METABOLISM.

III. THE EFFECT OF FAT AND FATTY ACID DERIVATIVES.

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(Received for publication, June 20, 1917.)

The influence of fat on the mineral metabolism is of prime importance in the nutrition of growth. Steinitz has shown that in infants a high fat diet may lead to a negative balance of the alkalis and a reduction of the alkali earths. On a diet rich in cream the absorption of calcium dropped from 76 to 34 per cent.

Cronheim and Mueller have shown that in children on an ordinary milk diet the fecal calcium is not solely combined as soaps. Some of it is excreted in this form but there is a great excess in other compounds. In other words, on a moderately rich fat diet only a small amount of the calcium is removed by the fatty acid.

Bahrdt has confirmed the statement of Cronheim and Mueller.

Rothberg found that a nourishment rich in fat gave a negative calcium balance. In the same children Birk found that the magnesium balance was also negative.

Meyer says that the results of Steinitz and Rothberg and Birk are not contradictory, but that the condition of the child greatly influences the results; as, for instance, if there were a diarrhea there be would less chance for absorption in the intestines.

In experiments referred to in Paper II of this series Kochmann found that addition of lard or dextrose to a diet of protein alone increased the urinary calcium excretion. From his study of the influence of protein, fat, and carbohydrate he concluded that calcium balance was dependent upon the kind and amount of nourishment.

Experiments which were being conducted in the laboratory by Dr. J. F. Lyman on the utilization of certain fats, fatty acids, and their derivatives offered a further opportunity to study the calcium excretion under these conditions.

Two male dogs were used, so that all experiments were made in duplicate. In the control periods the dogs received a standard diet of 250 gm. of clean beef, 50 gm. of cracker meal, 40 gm.

TABLE VII.*

Nitrogen and Calcium Metabolism, Averages per Day.—Dog J.

Period	Substituted for lard.	Urine.			Feces.		Balance.		Fat utilization.**
		N	Ca	Mg	N	Ca	N	Ca	
		gm.	*mg.*	*mg.*	*gm.*	*mg.*	*gm.*	*mg.*	*per cent*
1	——	8.83	19	18	0.60	257	+1.00	−171	95
2	Ethyl palmitate.	8.68	20	18	1.33	393	+0.41	−314	57
3	——	9.61	32	24	0.69	329	+0.07	−257	97
4	Glyceryl palmitate.	7.99	23	25	0.51	364	+1.93	−214	93
5		9.97	34	30	0.65	329	−0.19	−264	93
6	Palmitic acid.	9.15	27		0.76	472	+0.52	−400	81
7		8.89	31		0.66	72	+0.88	−485	97
8	Glyceryl palmitate + Ca lactate.	11.74	27		0.52	122	−1.82	+107	92
9		9.08	21		0.62	307	+0.73	−228	98
10	Starch and sucrose.	10.23	16		0.61	221	−0.44	−168	

* The tables are numbered consecutively through the three papers.

** My thanks are due Dr. J. F. Lyman for the use of these unpublished figures.

TABLE VIII.

Nitrogen and Calcium Metabolism, Averages per Day.—Dog FL.

Period.	Substituted for lard.	Urine.			Feces.		Balance.		Fat utilization.*
		N	Ca	Mg	N	Ca	N	Ca	
		gm.	*mg.*	*mg.*	*gm.*	*mg.*	*gm.*	*mg.*	*per cent*
1		10.12	11	20	0.70	300	−0.40	−214	94
2	Ethyl palmitate.	9.08	17	22	0.91	285	+0.44	−207	48
3		9.47	29	25	0.84	336	+0.15	−264	96
4	Glyceryl palmitate.	8.68	29	35	0.62	350	+1.13	−278	93
5		9.14	33	33	0.34	378	+0.64	−314	93
6	Palmitic acid.	9.25	29		0.67	492	+0.50	−428	79
7		9.84	37		0.76	705	+0.17	−700	95
8	Glyceryl palmitate + Ca lactate.	9.11	26		0.64	1,380	+0.68	−50	88
9		9.11	31		0.53	307	+0.79	−236	98
10	Starch and sucrose.	8.52	26		0.77	336	+1.13	−257	

* My thanks are due Dr. J. F. Lyman for the use of these unpublished figures.

of lard, 10 gm. of agar, and 400 cc. of tap water. In the other periods an equal quantity of ethyl palmitate, glyceryl palmitate, palmitic acid, and glyceryl palmitate plus calcium lactate (8 gm. daily for 2 days, then 10 gm. daily for 2 days) respectively were substituted for the lard. In the last period the caloric equivalent of 40 gm. of fat was replaced by a mixture of cooked starch and sucrose. The results are summarized in Tables VII and VIII.

The present conception of the digestion and utilization of fats and other comparable esters of fatty acid would lead one to expect that if they are hydrolyzed in the normally functioning alimentary tract the resulting fatty acid will either be absorbed promptly or excreted as insoluble soap with the feces. The extent to which absorption occurs may therefore depend not only upon the digestion of the esters but also upon the degree to which alkali earths are simultaneously present in the intestine to render the fatty acids insoluble and unutilizable. Conversely the loss of alkali earths through the bowel may likewise be promoted by the presence of large quantities of fatty acids. The extent of digestion and utilization of palmitic acid and its derivatives in Dr. Lyman's experiments will be recorded elsewhere. A study of the data here presented shows, with respect to the deportment of the calcium, that when the utilization is poor the loss of calcium is proportionately larger. This is exemplified as a rule in the following data selected from the tables.

TABLE IX.

Relation of Calcium Excretion to Fat Utilization.

	Fati n food.	Fat utilization.	Daily Ca output in feces.
		per cent	*mg.*
Dog J.	Lard.	95	257
	Ethyl palmitate.	57	393
	Lard.	93	329
	Palmitic acid.	81	472
Dog F.	Lard.	94	300
	Ethyl palmitate.	48	285
	Lard.	93	378
	Palmitic acid.	79	492

Similarly the negative calcium balance was smaller in those cases where the utilization of the fat was more satisfactory.

Although the quantity of calcium fed as calcium lactate in one of the periods was undoubtedly sufficient to induce a storage of lime on the basal diet (Table VII, Period 8) this could not be accomplished when the fat utilization was poor (Table VIII, Period 8).

It is evident from the data presented that poor utilization of fats or fatty acids may increase the excretion of lime in the feces and prevent the storage of calcium even when the calcium intake is comparatively abundant.

My thanks are due Professor Lafayette B. Mendel for his advice and criticism.

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THE RELATION OF THE QUALITY OF PROTEINS TO MILK PRODUCTION. III.*

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(Received for publication, July 2, 1917.)

In 1915[1] we presented data showing marked inequality in the efficiency of the protein mixture of rations for milk production. This difference in efficiency we attached to the character and quantitative proportion of the proteins constituting the mixture. We emphasized the fact that the mammary gland, in its constructive capacity for milk proteins, was not independent of the quality of the proteins in the ration and further that the "nutritive ratio" or plane of protein intake may be varied with variation in the source of the proteins. With milk proteins constituting about 70 per cent of the proteins of the ration for a cow—the remaining nitrogen coming from the roughage, corn stover—it was possible to maintain a positive nitrogen balance and the production of 35 pounds of milk per day with a nutritive ratio of 1:8. This is an exceptionally "wide" ratio. Where the proteins were drawn from the corn or wheat kernels and constituted the same proportion of the corn stover roughage ration as indicated above, no such positive nitrogen balances could be maintained. We pointed out that during the negative nitrogen balance increased tissue autolysis resulted, and for a brief time at least there was no decrease in the milk proteins or milk solids elaborated.

In 1916[2] we showed that with *corn stover* and corn meal as the basal ration there were appreciable differences in the efficiency

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[1] Hart, E. B., and Humphrey, G. C., *J. Biol. Chem.*, 1915, xxi, 239.

[2] Hart and Humphrey, *J. Biol. Chem.*, 1916, xxvi, 457.

with which certain protein concentrates could supplement such a ration for milk production. With the concentrates furnishing approximately 50 per cent of the total digestible proteins of the ration and the nutritive ratio fixed at approximately 1:8 (the total proteins constituted about 10 per cent of the dry matter of the ration) the gluten feed was measurably inferior to oil meal, distillers' grains, casein, or skim milk powder as a protein supplement in the particular mixture used. In the proportion used and with corn stover as the roughage none of these concentrates was able to maintain the animal in nitrogen equilibrium. A possible exception was milk powder. However, the distinctive point we wish to emphasize is that the negative nitrogen balance was much greater in the case of gluten feed than with the other materials used. The efficiency of the proteins of distillers' grains was attributed to the presence of the embryo of the seed.

We pointed out that the comparison would probably hold only for the mixture studied and that a different behavior might be expected should the basal ration be varied. Our expectations were entirely confirmed by the data to be presented. When the roughage of the ration was changed from corn stover to *clover hay* (medium red, *Trifolium pratense*) but the corn kernel maintained as the basal grain, the inefficiency of the gluten feed as a supplement disappeared, and among the four protein concentrates studied, namely, gluten feed, oil meal, distillers' grains (Ajax), and cottonseed meal there was little, if any, difference in efficiency. Furthermore, positive nitrogen balances were maintained during most of the periods of observation (16 weeks) on the nutritive ratio of 1:8.5.

EXPERIMENTAL.

The plan was to use a basal ration of clover hay, corn silage, corn meal, and starch, to which would be added the protein concentrate. The basal ration was maintained constant in relation to its source and proportion of nutrients for any individual in the different periods, the only variable in succeeding periods being the concentrate and the amount of starch. These were supplied in such quantities as to make the plane of protein intake and net available energy uniform in the several periods. After 16 weeks of observation with the various concentrates fed at a nutritive

plane of 1:8.5 the nutritive ratio was increased to approximately 1:5 for a period of 3 weeks by the addition of casein. This was done for the purpose of noting the effect of a high protein intake on both the quantity of milk secreted and its composition. This casein addition raised the total protein intake from approximately 12 per cent of the dry matter of the ration to 16 per cent.

Table I illustrates the proportion of the various feeds in the ration when an animal was receiving daily approximately 50 pounds of material.

TABLE I.

Source and Proportion of Nutrients Used.

Period	Gluten feed.	Distillers' grains.	Oil meal.	Cottonseed meal.
	lbs.	*lbs.*	*lbs.*	*lbs.*
Clover hay	8	8	8	8
Corn silage	28	28	28	28
Corn meal	6	6	6	6
Concentrate	4	3.37	3	2.88
Starch	4	4.63	5	5.12

Three Jersey cows of good milking capacity were used. Two were pure bred and one a grade. They were not with calf. The animals were milked twice daily and exercised two or three times a week. Their weight was taken weekly. The plan was to place each animal on any one of the rations for a period of 4 weeks with an immediate change to one of the other rations, thus involving each animal in 16 to 20 weeks of observation. A feeding period of 1 week preceded the quantitative collection of urine and feces.

The urine and feces were analyzed daily for nitrogen, while a weekly analysis was made of a 7 day composite sample of milk.

Our earlier observations had shown that when a nutritive ratio of 1:8 was used (equivalent to a plane of digestible protein intake of approximately 7 per cent, or 9 to 10 per cent of total protein) and the nutrients were drawn from corn stover, corn meal, and certain protein concentrates, a positive nitrogen balance could not be maintained. The daily production of milk in these earlier records was 35 to 40 pounds. Because of these facts it was planned to use rations with a nutritive ratio of 1:8 as it was essential that

TABLE II.

Composition of Rations.

	Weight.	Nitrogen.	Total N.	Production.	Digestible protein; nutritive ratio.
Gluten feed ration.					
	lbs.	*per cent*	*gm.*	*therms*	
Clover hay	8	1.90	69.00	2.75	
Corn silage	28	0.38	48.00	4.64	
Corn meal	6	1.65	44.90	5.38	2.2 lbs. digestible protein.
Gluten feed	4	3.98	70.20	3.17	
Starch	4	0.06	1.00	4.00	
Total	50		233.10	19.94	1:8.5
Distillers' grains (Ajax) ration.					
Clover hay	8	1.90	69.00	2.75	
Corn silage	28	0.38	48.00	4.64	
Corn meal	6	1.65	44.90	5.38	
Distillers' grains	3.37	4.65	71.40	2.66	
Starch	4.63	0.06	1.30	4.63	
Total	50		234.60	20.06	1:8.5
Oil meal ration.					
Clover hay	8	1.90	69.00	2.75	
Corn silage	28	0.38	48.00	4.64	
Corn meal	6	1.65	44.90	5.38	
Oil meal	3	5.25	71.50	2.36	
Starch	5	0.06	1.36	5.00	
Total	50		234.76	20.13	1:8.5
Cottonseed meal ration.					
Clover hay	8	1.90	69.00	2.75	
Corn silage	28	0.38	48.00	4.64	
Corn meal	6	1.65	44.90	5.38	
Cottonseed meal	2.88	5.47	71.44	2.18	
Starch	5.12	0.06	1.40	5.12	
Total	50		234.74	20.07	1:8.5
Oil meal-casein ration.					
Clover hay	8	1.90	69.00	2.75	
Corn silage	28	0.38	48.00	4.64	
Corn meal	6	1.65	44.90	5.38	
Oil meal	3	5.25	71.50	2.36	3.0 lbs. digestible protein.
Casein	1	12.71	57.65	1.00	
Starch	4	0.06	1.00	4.00	
Total	50		292.05	20.13	1:5

the animals be in negative nitrogen balance or just in nitrogen equilibrium in studies of this character. In the final adjustment of our rations the nutritive ratio became fixed at 1 : 8.5. In these rations this ratio was equivalent to approximately 8 per cent of digestible protein and 12 per cent of total protein. The composition of the rations used is shown in Table II.

TABLE III.

Record of Nitrogen Balance, Milk Nitrogen, Etc.

Animal 1, Jersey.

Date.	N intake.	N feces.	N absorbed.	N urine.	N milk.	N balance.
Gluten feed period.						
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	
Dec. 5–11	1,514	699	815	271	537	+ 7
" 12–18	1,452	678	774	292	545	− 63
" 19–25	1,435	633	802	266	536	00
" 26–Jan. 1	1,435	655	780	267	517	− 4
Oil meal period.						
Jan. 2– 8	1,443	629	814	258	511	+ 45
" 9–15	1,443	591	852	233	524	+ 95
" 16–22	1,443	636	807	243	511	+ 53
" 23–29	1,443	680	763	242	523	− 2
"Ajax" period.						
Jan. 30–Feb. 5	1,443	693	750	227	514	+ 9
Feb. 6–12	1,443	702	741	243	485	+ 13
" 13–19	1,443	718	725	208	454	+ 63
" 20–26	1,443	718	725	224	457	+ 44
Cottonseed meal period.						
Feb. 27–Mar. 5	1,443	654	789	238	442	+107
Mar. 6–12	1,443	756	687	265	463	− 39
" 13–19	1,443	714	729	269	487	− 27
" 20–26	1,443	646	797	222	461	+114
Oil meal-casein period.						
Mar. 27–Apr. 2	1,847				476	
Apr. 3– 9	1,847				521	
" 10–16	1,847				496	

TABLE IV.

Record of Nitrogen Balance, Milk Nitrogen, Etc.

Animal 2, Jersey.

Date.	N intake.	N feces.	N absorbed.	N urine.	N milk.	N balance.
Gluten feed period.						
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	
Dec. 5–11	1,076	462	614	296	295	+23
" 12–18	1,020	453	567	323	278	−34
" 19–25	978	431	547	294	269	−16
" 26–Jan. 1	978	475	503	285	245	−27
Oil meal period.						
Jan. 2– 8	985	407	578	340	234	+ 4
" 9–15	985	396	589	265	244	+80
" 16–22	985	385	600	283	256	+61
" 23–29	985	385	600	273	267	+60
"Ajax" period.						
Jan. 30–Feb. 5	985	515	460	230	255	−15
Feb. 6–12	985	442	543	214	245	+84
" 13–19	985	477	508	225	232	+51
" 20–26	985	500	485	226	260	− 1
Cottonseed meal period.						
Feb. 27–Mar. 5	985	423	562	263	247	+52
Mar. 6–12	985	487	498	245	232	+21
" 13–19	985	484	501	230	240	+31
" 20–26	985	488	497	213	222	+62
Oil meal-casein period.						
Mar. 27–Apr. 2	1,388				236	
Apr. 3– 9	1,388				254	
" 10–16	1,388				253	

The first four rations were much alike in content of production therms and total protein. Table II gives the composition of 50 pounds of the mixed ration. The animals were not fed 50 pounds daily, but what proportion of the ration would meet their maintenance and the energy requirements for their normal milk production. The production energy of the rations allowed con-

formed closely to Armsby's standard of 7 therms daily for maintenance and 0.3 therm for each pound of 4 per cent milk produced by a 1,000 pound cow. For example, Animal 1 weighed 950 pounds and produced daily 30 to 35 pounds of milk containing 4 to 5 per cent of fat, and consumed 44 pounds of the ration. In 50 pounds of the first four rations used there were approximately

TABLE V.

Record of Nitrogen Balance, Milk Nitrogen, Etc.

Animal 3, Jersey.

Date.	N intake.	N feces.	N absorbed.	N urine.	N Milk.	N balance.
Oil meal period.						
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	
Dec. 5–11	1,181	473	708	210	478	+ 20
" 12–18	1,181	567	614	220	467	− 73
" 19–25	1,181	509	672	182	465	+ 25
" 26–Jan. 1	1,181	548	633	169	455	+ 9
Gluten feed period.						
Jan. 2– 8	1,181	477	704	207	453	+ 44
" 9–15	1,181	466	715	181	433	+101
" 16–22	1,181	455	726	193	441	+ 92
" 23–29	1,181	504	677	187	431	+ 59
"Ajax" period.						
Jan. 30–Feb. 5	1,181	548	633	176	415	+ 42
Feb. 6–12	1,181	544	637	159	398	+ 80
" 13–19	1,181	556	625	158	400	+ 67
" 20–26	1,181	541	640	144	386	+110
Cottonseed meal.						
Feb. 26–Mar. 5	1,181	484	597	193	370	+134
Mar. 6–12	1,181	659	522	197	381	− 56
" 13–19	1,181	539	642	193	382	+ 65
" 20–26	1,181	523	658	202	386	+ 70
Oil meal-casein period.						
Mar. 27–Apr. 2	1,584				404	
Apr. 3– 9	1,584				396	
" 10–16	1,584				457	

2.22 pounds of digestible protein, 40 per cent of which came from the particular concentrate under investigation.

In Tables III, IV, and V are recorded by weekly periods the nitrogen balances and the nitrogen secreted in the milk. The figures represent the intake and output for the total 7 days.

In addition to the tables, charts are added showing the positive and negative nitrogen balances and the gm. of nitrogen produced in the milk. There are also added charts illustrating the volume of the milk secreted weekly and its content of solids and fat.

A survey of the data indicates a very uniform behavior in nitrogen metabolism with the different protein mixtures. There was no sudden or increased excretion of urinary nitrogen with the concentrate gluten feed as was observed in our earlier work where corn stover formed the roughage. Evidently the proteins of clover hay will supplement the proteins of gluten feed in a much more efficient manner than will those of corn stover. The same uniformity of nitrogen metabolism prevailed with the other concentrates. *These results emphasize in a very striking manner the limitations of any classification of natural foods in respect to the efficiency of their proteins, based on the determination of such nutritive work in a single food material or single mixture.* When they are used in mixtures as they generally are, the efficiency may be very greatly modified by the supplementary materials. This applies to human as well as animal nutrition. Lusk's[3] classification of the proteins of foods into the groups A, B, and C, according to their physiological value, has significance only in so far as the individual food materials form the sole article of diet. In a mixture, Class A would probably always improve Class C, but it is possible to conceive of Class C proteins from independent sources making an efficient mixture.

Positive nitrogen balances were maintained by all the animals over most of the periods of observation, although the plane of protein intake was low as compared with that usually prescribed for milking animals. These animals not only maintained nitrogen equilibrium, but also maintained their live weight. Their initial weights were 952, 762, and 683 pounds respectively. At the termination of the experiment they weighed 923, 800, and

[3] Lusk, G., Fundamental Basis of Nutrition, New Haven, 1914.

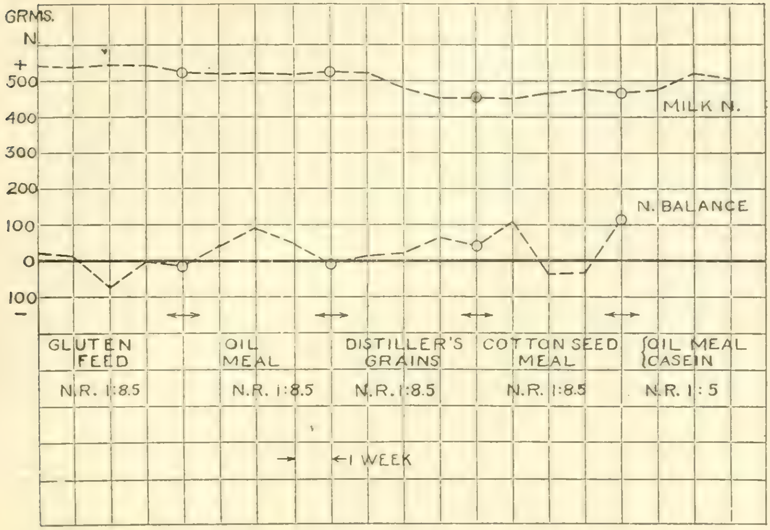

CHART 1. Animal 1. Showing the nitrogen balances with different sources of protein and the milk nitrogen production.

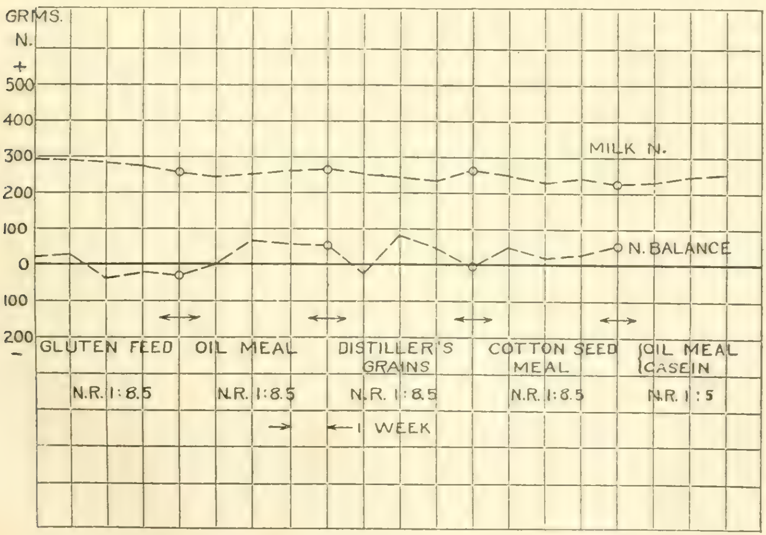

CHART 2. Animal 2. Showing the nitrogen balances with the different sources of protein and the milk nitrogen production.

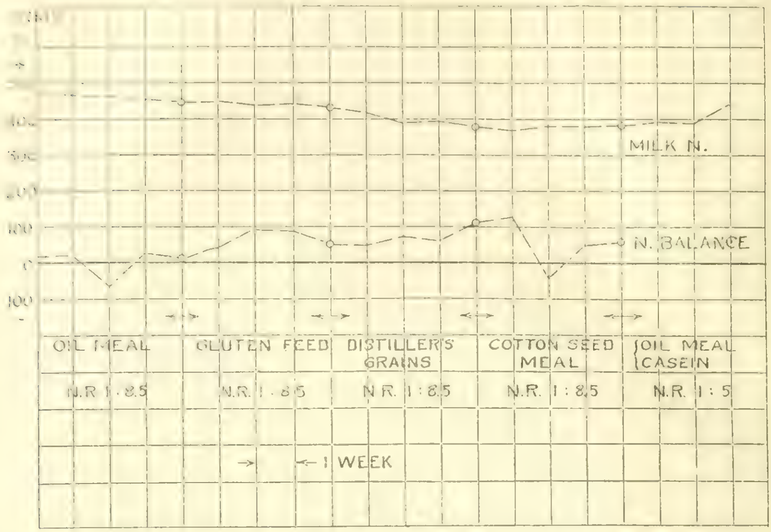

CHART 3. Animal 3. Showing the nitrogen balances with the different sources of protein and the milk nitrogen production. Attention should be called to the positive nitrogen balances maintained with gluten feed.

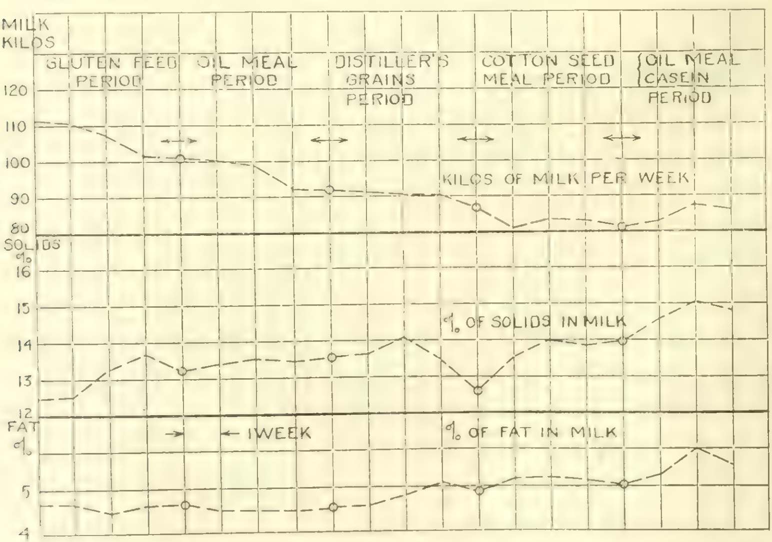

CHART 4. Animal 1. Showing the proportion of milk secreted and the solids and fat content of the milk.

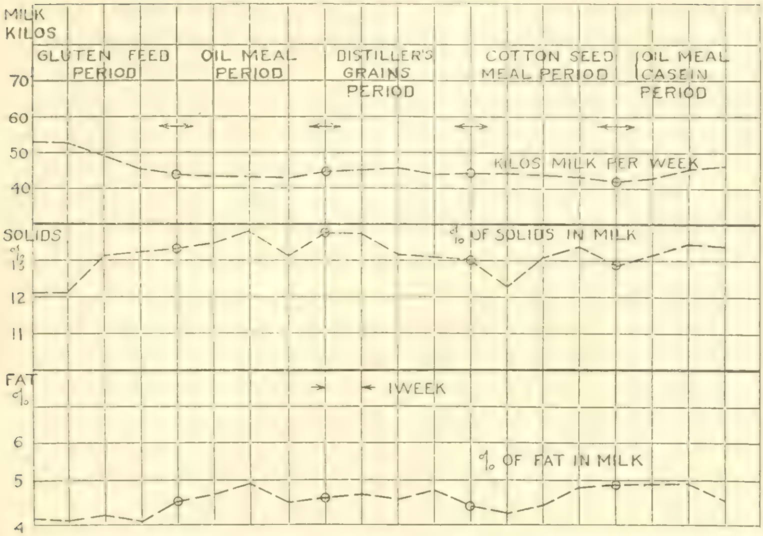

CHART 5. Animal 2. Showing the proportion of milk secreted and the solids and fat content of the milk.

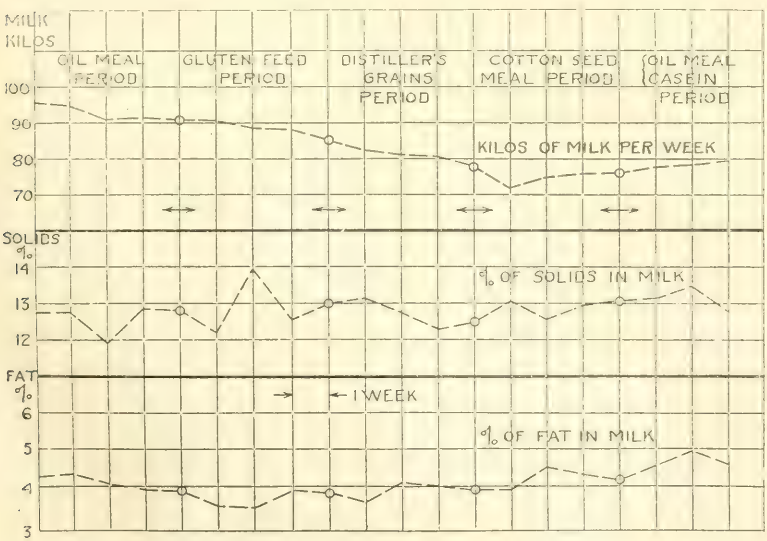

CHART 6. Animal 3. Showing the proportion of milk secreted and the solids and fat content of the milk.

689 pounds respectively. While all these animals were of strong dairy type and normally somewhat thin in flesh there was no evidence that they were more emaciated at the end of the experimental period than at its beginning. It is apparent that where ample net available energy is provided in a ration, a plane of protein intake may be found which will be lower than that prescribed by the standards and which will at least maintain nitrogen equilibrium, live weight, and a fairly well sustained flow of milk.

But the maintenance of nitrogen equilibrium, under such conditions, will depend upon the mixture of proteins used. Such nitrogen equilibrium as obtained in these experiments was impossible with any of the seed protein concentrates investigated when corn meal and *corn stover* formed the basal portion of the ration and were fed at a level of 1: 8.

The mere maintenance of nitrogen equilibrium on the lower plane of protein intake and with selected materials did not, however, suffice for a sustained flow of milk. There was a gradual shrinkage in volume as the duration of the experiment progressed. The shrinkage was not large and was greater with some individuals than with others. Animal 1 was producing 35 pounds of milk daily when the experiment was initiated and 25 pounds before the period of high protein feeding; No. 2, 17 pounds at the beginning of the experiment and 13 pounds before the period of high protein feeding; No. 3, 31 pounds at the beginning of the experiment and 24 pounds before the period of high protein feeding. All showed a shrinkage in volume, but there was a maintenance of the percentage of total solids, fats, and proteins in the milk. But even with the maintenance of the percentage composition the shrinkage of volume means that there was actually a slow decrease in the elaboration of all classes of compounds by the mammary gland. In a previous publication[2] we presented data showing that a milch cow in negative nitrogen balance withdrew liberally from her own tissues, for a time at least, and continued to elaborate milk proteins; but under such conditions there was not only a slow shrinkage in volume of milk produced, but an actual decrease in the percentage composition of the milk. In the experiment reported in this paper where nitrogen equilibrium was maintained, but the protein level of intake was low, shrinkage of flow followed, but the percentage composition was maintained.

To show these effects the composition and volume of the milk secreted by the several animals at different periods of observation are given in Table VI.

TABLE VI.

Decrease in Milk Volume, but Maintenance of Percentage Composition on Low Protein Intake, but in Nitrogen Equilibrium.

Animal No.		Dec. 15.	Jan. 12.	Feb. 9.	Mar. 9.	High protein, Apr. 6.
1	Total solids, *per cent*............	13.40	15.60	14.10	14.00	15.10
	Fat, *per cent*....................	4.30	4.40	4.80	5.30	6.00
	Nitrogen, *per cent*...............	0.51	0.51	0.53	0.53	0.57
	Milk daily, *lbs*..................	33.60	31.70	28.80	27.10	28.20
2	Total solids, *per cent*............	13.30	15.90	13.20	13.30	13.50
	Fat, *per cent*....................	4.20	4.90	4.50	4.40	4.90
	Nitrogen, *per cent*...............	0.57	0.51	0.54	0.56	0.54
	Milk daily, *lbs*..................	15.00	14.40	14.70	13.90	14.50
3	Total solids, *per cent*............	11.90	14.00	12.70	12.60	13.50
	Fat, *per cent*....................	4.00	3.90	3.90	4.40	4.50
	Nitrogen, *per cent*...............	0.50	0.46	0.47	0.47	0.51
	Milk daily, *lbs*..................	28.20	27.00	25.30	24.30	23.00

In the period of high protein feeding there was not only a stimulation to flow, but an actual increase in solids secreted. The peculiar stimulating effect of liberal protein feeding on mammary activity was strikingly shown in these records. The maintenance of milk flow desired by every dairyman is very probably secured by his customary high protein feeding, but at what expense is not so clear. Whether we could have partly prevented the shrinkage of milk flow observed in these experiments by the use of better or higher protein intake cannot be answered, but in practice where a high protein level is used there is also a decreased flow of milk incident to an advancing lactation. In view of the fact that there was an acceleration of mammary gland activity during the period of high protein feeding it is probably correct to attribute some of the milk shrinkage observed in this work to a low protein intake. From the data in Table VII it should be noted that if we include the tissue nitrogen storage with that of the milk there was not an appreciable decrease in nitrogen utili-

zation for the combined process of milk production and tissue reparation with advancing lactation. A redistribution of the nitrogen between milk and tissue was slowly in progress.

Efficiency of Protein Mixtures Compared.

The basis for comparing the efficiency of these protein mixtures involves not only the milk proteins elaborated, but in addition the protein catabolized or stored during the periods of observation. We had expected that our animals would be in negative nitrogen balance on the low protein level used, but for most of the periods positive nitrogen balances prevailed. The fact that the storage of nitrogen was in any case but slight while the flesh condition of the animals would probably have allowed more ample protein storage had it been available makes it evident that we were in all cases approximately close to nitrogen equilibrium. While it is necessary that negative nitrogen balances prevail for measuring the efficiency of the protein mixture for milk production, particularly where the animal is in good flesh, yet it is possible to make accurate measurements of this efficiency if the animal is in positive nitrogen balance, but in poor flesh. This then involves the protein in both milk production and tissue building. For the reason that our animals were rather thin in musculature and that positive nitrogen balances were at most but slight we have confidence in the results recorded. For purposes of making definite comparisons we have calculated the percentage of efficiency for the various mixtures used on the basis of absorbed nitrogen, tissue destroyed or stored, and milk proteins produced. Manifestly the absorbed nitrogen should be used in the calculation rather than the total nitrogen ingested. In Table VII these comparisons are made.

The average percentage of efficiency was very uniform with the different concentrates and the same individual. Much the greatest variation was among the individuals. Chart 7 illustrates these differences. Animal 2 showed an *average* efficiency on all protein mixtures of but 51 per cent, with the lowest on gluten feed (46 per cent) and the highest on distillers' grains (55 per cent); while No. 3 showed on the same rations on *average* efficiency of 72 per cent, with the highest efficiency (75 per cent) on distillers' grains and the lowest (70 per cent) on oil meal.

TABLE VII.

Relative Efficiency for Milk Production of Protein Mixtures Involving Gluten Feed, Distillers' Grains, Oil Meal, and Cottonseed Meal.

Animal No	Date.	Ration	N absorbed.	N in milk + tissue N formed or destroyed	Efficiency.
			gm.	*gm.*	*per cent*
1	Dec. 5–Jan. 1	Gluten feed.	3,171	2,075	65
	Jan. 2–29	Oil meal.	3,236	2,260	69
	" 30–Feb. 26	Distillers' grains.	2,941	2,039	69
	Feb. 27–Mar. 26	Cottonseed meal.	3,002	2,008	66
2	Dec. 5–Jan. 1	Gluten feed.	2,231	1,033	46
	Jan. 2–29	Oil meal.	2,367	1,205	50
	" 30–Feb. 26	Distillers' grains.	1,996	1,111	55
	Feb. 27–Mar. 26	Cottonseed meal.	2,058	1,107	53
3	Dec. 5–Jan. 1	Oil meal.	2,627	1,846	70
	Jan. 2–29	Gluten feed.	2,822	2,054	72
	" 30–Feb. 26	Distillers' grains.	2,535	1,898	75
	Feb. 27–Mar. 26	Cottonseed meal.	2,419	1,732	71

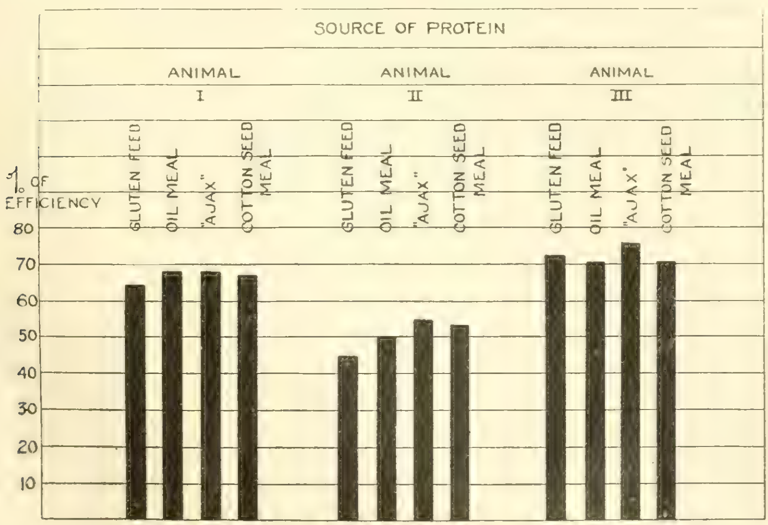

CHART 7. Showing the comparative efficiency for milk production of the different protein mixtures. Note the uniformity of any one animal with the different mixtures, but the large individual variation.

SUMMARY.

Data are presented on the comparative value for milk production of protein mixtures involving gluten feed, oil meal, distillers' grains, and cottonseed meal.

These concentrates furnished approximately 40 per cent of the digestible protein of the ration and were used to supplement a basal ration of corn meal, corn silage, and clover hay. The total protein intake constituted about 12 per cent of the dry matter of the ration and the nutritive ratio was approximately 1 : 8.5.

On this low protein intake positive nitrogen balances were maintained during most of the period of observation (16 weeks) with a slow shrinkage in milk volume, but a maintenance of the percentage composition of the milk.

Earlier records showed the inferiority of the proteins of gluten feed as a supplement to the proteins of corn meal and *corn stover* for milk production to those of oil meal, distillers' grains, or milk. These records show an equality in efficiency between the proteins of gluten feed, oil meal, distillers' grains, and cottonseed meal as supplements to the proteins of corn meal and *clover hay* for milk production.

These facts must emphasize in a very striking manner the limitations of any classification of natural foods in respect to the efficiency of their proteins, based on the determination of such nutritive worth in a single food material or a single food mixture.

A STUDY OF THE EFFECT OF HYDROCHLORIC ACID ON THE MINERAL EXCRETION OF DOGS.*

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It is conceivable that the administration of an unoxidizable acid to a normal living organism or the formation of such an acid by the organism itself may exert a deleterious action (1) by neutralizing alkali necessary for the transportation of carbon dioxide formed in the metabolic processes; (2) by direct toxic action or by the toxic action of a resulting salt; (3) by diminishing tissue alkalinity and thereby influencing metabolic activities (this change may be in the potential alkalinity rather than in the actual hydroxyl ion concentration, the effect then being due to a diminution in the cation concentration below that which is essential for normal metabolism). To determine which of these three possibilities actually occurs has been the basis for numerous investigations, the ultimate object being to explain the coma of diabetes mellitus. Experimental evidence has been obtained in support of each.

HISTORICAL.

To determine whether the body is depleted of its stock of alkali necessary for the transportation of carbon dioxide several methods have been employed. They have been direct and indirect, the latter type predominating. Where the direct method—the quantitative determination of the bases excreted—has been employed it has not been carried out in detail, that is, the bases have not been determined individually. Carnivora and

* The work here reported was undertaken jointly with Dr. A. E. Taylor but the utilization of the latter's time by the national government has made his participation impossible. The author desires to express his thanks to Dr. Taylor, however, for placing his own previous experience in connection with the problem at the writer's disposal.

herbivora have been used and the results seem to indicate that much depends upon the dietary habits of the experimental animals. Salkowski (1873), using rabbits and feeding taurine with the expectation that it would be oxidized to sulfuric acid, observed an increase in both the sodium and potassium outputs. He was led by the variation of his own results from the earlier negative results of Gaethgens (1872), Eylandt (1854), and Wilde (1855), obtained with dogs and men, to postulate a difference between carnivora and herbivora in the matter of alkali excretion after acid administration. Salkowski did not determine the ammonia excreted and it remained for Walther (1877) to discover that in the dog the acid was excreted to a great extent as the ammonium salt. For this reason Walther believed dogs to be much more resistant to acids than rabbits whose death he explained as due to the withdrawal of alkali necessary for the transportation of carbon dioxide. Walther's figures showing an enormous decrease in the carbon dioxide content of the blood give credence to this belief. Eppinger (1906) has reported experiments intended to settle the question of a difference between carnivora and herbivora as regards resistance to acid. His results were such as to justify the conclusion that rabbits ordinarily react differently from dogs merely because their diet is low in protein. By proper protein feeding he was able to increase the resistance of rabbits to acid and by administering amino-acids or urea simultaneously with the acid he found that the symptoms of coma did not appear. Pohl and Münzer (1906), Pohl (1909), and Bostock (1913) dispute the correctness of Eppinger's results though Eppinger and Tedesko (1909) have repeated and extended the former's experiments. The names of Auerbach (1884), Winterberg (1898), Kettner (1902), Spiro (1902), and Labbé and Violle (1911) should be mentioned in connection with this phase of acid intoxication studies.

Of the direct methods for determining the effect of acid administration upon the withdrawal of alkalies the determination of the carbon dioxide content of the blood and of the latter's capacity to neutralize acid have been employed most frequently. Parallel analyses have been made by many investigators. The results of most of the experiments reported show that in acid intoxication both carbon dioxide content and titratable alkalinity are lowered. Lassar (1874), Kraus (1889, 1890), Loewy and Münzer (1901), Spiro (1902), and Landau (1905) have helped develop this aspect of the problem.

Low carbon dioxide values are by no means confined to acid intoxication. They have been found to follow the administration of phosphorus, iron, arsenic, emetin, and many other substances (Meyer and collaborators, 1881, 1883), in fever (Kraus, 1889), after the administration of sodium butyrate and sodium isobutyrate (Loewy and Erhmann, 1911), etc. In diabetes Beddard, Pembrey, and Spriggs (1904) found the carbon dioxide content and acid neutralizing power diminished. They found also, however, that the blood had not lost its power to bind carbon dioxide. By tying off the arm of a diabetic they observed the carbon dioxide content to be increased from 40 to 45 volumes per cent to 61.8 volumes per cent. The low carbon

dioxide values are attributed by them to a stimulation of the respiratory center by acids other than carbonic and to a diminished carbon dioxide production.

Some attempt has been made to measure the effect of acid on the blood alkali by measuring the hydrogen ion concentration after acid administration and by a study of the blood of diabetics. Szili (1906), by intravenous injections of acids into dogs, found the hydrogen ion concentration decreased. Benedict (1906), Rolly (1912), and Poulton (1916) found the hydrogen ion concentration increased in some diabetics and unchanged in others.

The significance of a low carbon dioxide content of the blood is detracted from by the results of Beddard, Pembrey, and Spriggs, already mentioned. Data given by Benedict and Joslin (1910) do not support the low alkali theory of diabetic coma any better. These authors found the total metabolism of diabetics to be somewhat higher than that of normal individuals but their data show respiration and pulse rate to be at least as low as normal, which would indicate that the body is experiencing no difficulty in eliminating its carbon dioxide. If the hydrogen ion concentration is the controlling factor in respiration a deficiency of alkali should surely show itself by the action of the increased amount of carbon dioxide in solution on the delicate mechanism which the respiratory center is supposed to be. The damming up of carbon dioxide in the tissues should also show itself in an increase in the carbon dioxide content of the urine in severe diabetes but such does not occur according to Beddard, Pembrey, and Spriggs.

The hypothesis that diabetic coma is attributable to toxic action has found some supporters. It is sufficient here to call attention to the work of Mayer (1886), Wilbur (1904), Marx (1910), and Ehrmann and his collaborators (1911).

Inasmuch as there are no complete data on the excretion of the metallic elements during acid intoxication the third hypothesis referred to above has little to support it. Chvostek (1893) found the oxygen utilization, carbon dioxide production, and heat formation lowered during acid intoxication. In agreement with these results are some older experiments of Munk (1881) which showed that after long treatment with hydrochloric acid the high oxidizing capacity of the horse for phenol was reduced 41.2 per cent. The results of the present investigation furnish evidence for believing that diabetic coma may *possibly* be a result of the withdrawal of essential cations.

EXPERIMENTAL.

No detailed study has been made, as mentioned above, of the excretion of bases after acid administration. It seemed desirable, therefore, to subject this phase of the problem of acid intoxication to a fuller investigation.

A female dog weighing between 9 and 10 kg. served for the two experiments to be reported here. The diet consisted of 50 gm. of soda crackers,[*] 150 gm. of beef heart, and 10 gm. of agar-agar intimately mixed and containing carmine every other day. The acid. in the first period of the series was given in the form of glycocoll hydrochloride (enclosed in balls of food) and in the second period as dilute hydrochloric acid (0.125 N and 0.250 N). When dilute acid was employed the same volume of water was given on the control days. No difficulty was experienced by the animals in retaining the amounts administered. Chlorinated fat was tried with the hope that by its use large doses of potential hydrochloric acid might be given in small volume but the material employed which contained 21 per cent chlorine was not retained by the animals. Very small quantities were vomited. 24 hour samples of urine were collected by catheterization at the end of each experimental day, that portion which was voided spontaneously being collected over chloroform. The feces were separated as nearly as possible into portions corresponding to the food from which they came but the analytical results indicate that such a procedure does not give as clear a picture of the excretion of bases as is obtainable from the urine. Average figures for the various periods are more significant in the case of the feces.

Analytical Methods.

Urine and feces were ashed in the wet way and the solution then evaporated to dryness. For determining the sodium and potassium the residue was heated with dilute hydrochloric acid and the solution then precipitated with saturated barium hydroxide solution until the reaction was alkaline. The precipitate was separated with the aid of the centrifuge and the excess barium precipitated with sulfuric acid. The filtrate on evaporation gave combined sodium and potassium sulfates. (In the case of feces the sulfuric acid solution contained some calcium which was removed by precipitation in ammoniacal solution with ammonium oxalate. Potassium alone was then estimated by precipitating it as potassium sodium cobaltic nitrite and titrating the nitrous acid with standard permanganate. The details of the method are described by Drushel (1908). Sodium was determined by

difference. The procedure for calcium and magnesium was the ordinary oxalate and pyrophosphate method. In the case of urine known amounts of calcium and magnesium were previously added in order to be better able to judge of the satisfactoriness of the precipitation.

DISCUSSION.

Chart I shows the variations in the potassium excretion during the 26 days of the experiment. The normal potassium excretion on the diet employed is close to 400 mg. per day. The administration of 9 gm. of glycocoll hydrochloride caused the output to rise immediately, but it is noteworthy that even while the hydro-

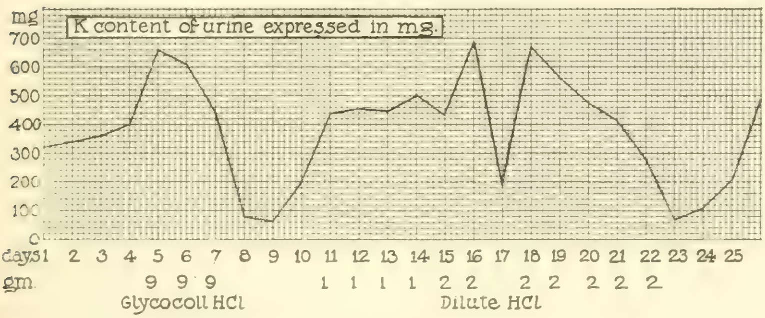

Chart I.

chloride was still being administered the excretion began to fall and on the 8th day when none was given the excretion was only one-fourth of the normal amount. On the 10th day the rate of excretion was again approaching the normal but rose noticeably on subsequent days when free hydrochloric acid was given. The same subnormal excretion, when the administration of acid was stopped, and even before, is again apparent. It would appear as though the experience of a day or two enabled the animal to combat successfully the attempt to withdraw its potassium supply and even to recover by subnormal excretion what was lost originally by the administration of acid.

Chart II, showing the sodium excretion, is less clear. The maxima of the curves parallel those of the potassium excretion,

however, and it is probably true that the same phenomenon occurs here though to a less pronounced degree.

These results are difficult to reconcile with the conclusions based upon the determinations of blood carbon dioxide and titratable alkalinity. The results of such determinations seem to require a loss of alkali but evidence of its elimination does not appear here.

The calcium and magnesium contents of the urine are almost insignificant, say 20 mg. of each on the diet employed. Chart

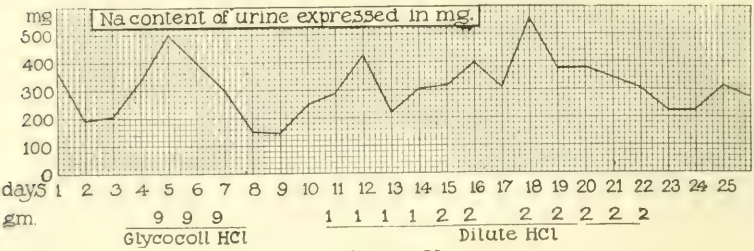

Chart II.

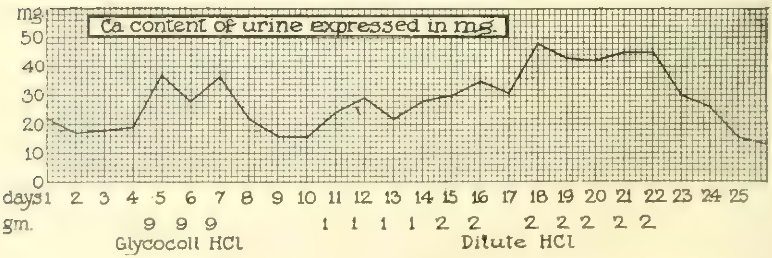

Chart III.

III shows, however, that the same variations occur here as in the case of potassium with possibly a less pronounced tendency to recover lost base after the administration of acid has ceased. The magnesium excretion, Chart IV, bears the same relation to the calcium excretion that the sodium excretion does to the potassium. It can hardly be denied that there is a slight tendency for the magnesium excretion to increase during the administration of acid.

The results of the urine analyses are shown collectively in Chart V. Here the quantities are plotted in gm. equivalents ×

10,000 instead of in mg., with the result that the relative rôles in neutralizing the acid administered are made apparent. It may be seen that when so plotted the variations in the calcium and magnesium excretions are hardly perceptible. The variations in

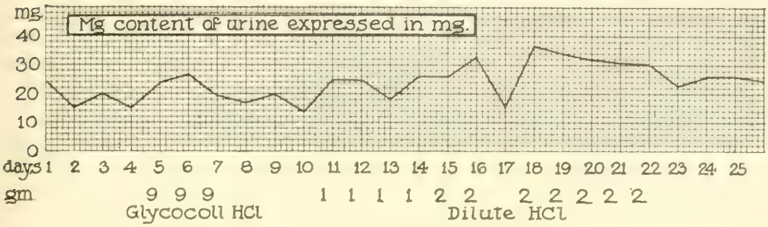

CHART IV.

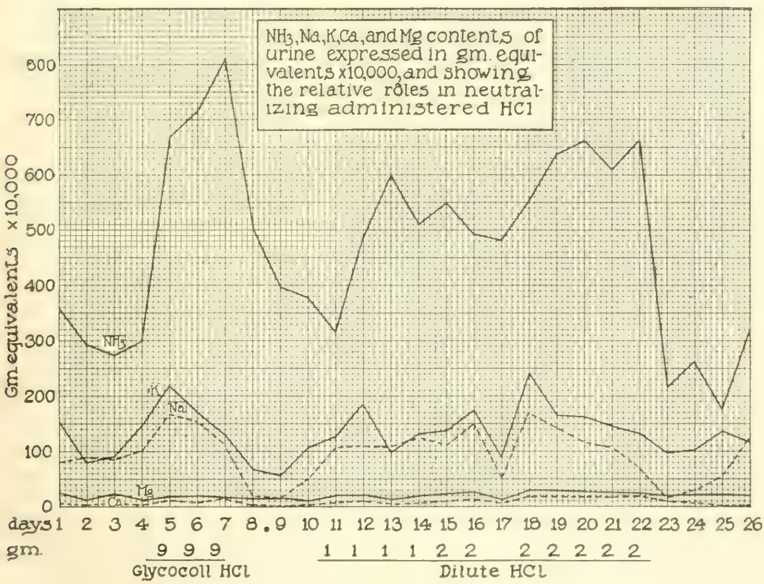

CHART V.

the potassium and sodium outputs are plainly in evidence but they are small in comparison with the changes in the ammonia output. This base is the great factor in neutralizing the acid administered.

As was stated above, the analysis of the feces corresponding to the food of each of the 26 days of the experiment does not give results which make their plotting individually very significant. However, it is not to be expected that the feces corresponding to the food of a day on which acid was given will necessarily reflect the effect of that acid. It may be that the excess of base excreted will appear in the feces corresponding to the food of an earlier day and indeed some of the results seem to indicate as much. In addition it was impossible to separate the feces sharply even when marked as they were and some of the fluctuations are attributable to this fact. By taking average excretions of the

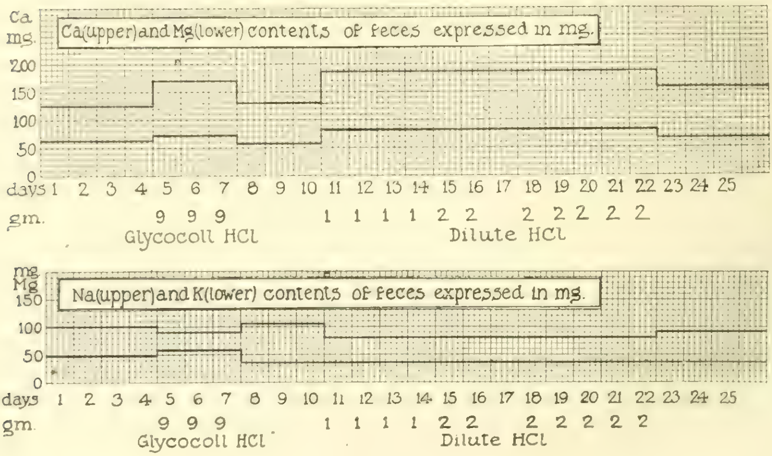

Chart VI.

different periods, however, figures are obtained which when plotted give Chart VI. There can be no doubt that both the calcium and magnesium excretions are increased to a greater extent than in the urine. This is not true of the sodium and potassium, and it is a fair conclusion that the results in this case do not indicate any loss of these two elements through the intestine during acid administration. It may be that a more detailed study would show an initial rise followed by a compensating fall.

It seems right to conclude from the results obtained that calcium and to a less extent magnesium are the only metals lost by acid administration. Taking the normal calcium excretion in the

present case as 130 to 140 mg. per day in the feces and 20 mg. in the urine, a calculation shows that the administration of 2 gm. of hydrochloric acid causes an additional excretion of about 75 mg. Now 1 gm. of hydrochloric acid is equivalent to 2.85 gm. of β-hydroxybutyric acid, and if we consider a 70 kg. man instead of a 10 kg. dog, the corresponding β-hydroxybutyric acid excretion would be 40 gm. This is close to the maximum excreted in severe diabetes. When such a quantity of this acid is being eliminated the loss of calcium would amount to 0.53 gm. In one of the cases of diabetes reported by Benedict and Joslin (1910) β-hydroxybutyric acid determinations were made frequently and in the last 160 days of the patient's life calculation shows that somewhat more than 5 kg. of β-hydroxybutyric acid were excreted. Inasmuch as it was just found that 40 gm. of the acid corresponds to 0.53 gm. of calcium, the loss of this element during the 160 days would amount approximately to 66 gm. Such an amount does not seem large when the whole calcium content of the body is considered but it is unknown, of course, to what extent the calcium of the body must be considered as inert. If the quantity is large then 66 gm. may be quite a significant loss. The analogy between man and the dog may not be complete and perhaps is not. It is likely, for example, that because of man's lower protein diet ammonia may play a less important rôle in neutralizing ammonia than it does in the dog, with the result that the loss of other cations would be augmented and the harmful effects increased.

CONCLUSIONS.

The administration of hydrochloric acid by mouth to the dog causes an increased excretion of calcium and magnesium as well as of sodium and potassium but in the case of the latter pair a compensatory retention makes the loss apparent rather than real.

If an analogous condition holds in human diabetes the resulting calcium loss may be something to take into consideration in the treatment of diabetic patients in whom the excretion of hydroxybutyric acid has reached a significant figure.

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THE RELATION OF ADRENALIN HYPERGLYCEMIA TO DECREASED ALKALI RESERVE OF THE BLOOD.

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(Received for publication, June 25, 1917.)

During the course of some attempts by one of us to increase the food tolerance of diabetic patients by subcutaneous injections of adrenalin, it was observed that one of the patients showed more or less pronounced hyperpnea immediately after the administration of adrenalin. This observation, taken in connection with the fact that lowering of the alkaline reserve of the blood is often accompanied by some degree of hyperpnea, led to the investigation of the effect of adrenalin upon the CO_2 combining capacity of the blood. The fact that adrenalin also causes hyperglycemia, that the administration of acid increases this hyperglycemia (1), and as pointed out by Elias (2), that the intravenous injection of acid alone causes hyperglycemia, led to the suspicion that adrenalin causes hyperglycemia by lowering the alkaline reserve of the blood. Therefore to investigate this hypothesis parallel estimations of blood sugar and blood CO_2 combining capacity have been made after administration of adrenalin in cases of diabetes and in normal individuals.

To complete the study, observations on alveolar CO_2 tension, blood pressure, pulse rate, and glycosuria are added.

Three cases of diabetes were chosen because of the ease in which rapid and extreme changes in blood sugar can be produced. Later two normal men were also studied in the same way, showing that the changes observed were not peculiar to diabetics.

The procedure was as follows: All the cases were studied fasting, the periods of fasting before the administration of adrenalin ranging from 11 to 41 hours. From 15 to 30 minutes before the administration of adrenalin and at varying intervals thereafter, blood was obtained for estimation of the plasma CO_2 combining

capacity and for sugar analysis. Samples of alveolar air were taken for estimation of the CO_2 tension. Blood pressure and pulse rate were also determined.

Adrenalin 1:1,000 (Parke, Davis and Company) was given subcutaneously in doses of 10 to 21 ℳ. Patients were carefully watched for appearance of symptoms, and the fast was continued to the end of the period of observation.

The blood sugar was determined by the method of Lewis and Benedict and the blood CO_2 by the Van Slyke method, the results being expressed in terms of alveolar air. Alveolar CO_2 tension was determined by the Fridericia method. The sugar in the urine was determined quantitatively and qualitatively by the Benedict methods. Charts and protocols follow at the end of the paper.

From a study of the charts it will be noted that in all the experiments the subcutaneous administration of adrenalin in doses ranging from 10 to 21 ℳ of the 1:1,000 solution produced a diminution in the blood CO_2 combining capacity synchronous with a rise in the blood sugar concentration. These changes occurred in all cases in varying degrees within the first half hour and reached their maximum intensity within 3 hours. They were followed within 6 to 8 hours by a drop in the blood sugar to its original level or to a level lower than that previously observed. The blood CO_2 combining capacity returned to its original level or above except in Cases 3 and 4, where within the experimental period the former level was not quite attained. We have not been able to explain this observation. In Case 5 the blood CO_2 returned to approximately its original level within the 1st hour but 4 hours later it was even higher than it had been in the first observation after adrenalin. In this case it will be observed that a relatively small dose of adrenalin was given, the reaction in every respect was comparatively slight, and as in Case 1, there was no sugar in the urine.

Alveolar CO_2.—The incompleteness of the observations as regards the alveolar CO_2 is due to the difficulty encountered in getting good samples of air in Cases 1 and 2, while in Case 3 the same difficulty obtained half an hour after adrenalin was given.

DISCUSSION.

It might be argued that the hyperpnea produced by adrenalin was not due to a change in the reaction of the blood and that the low values obtained for the plasma carbonates were due merely to a diminution of the CO_2 tension of the blood caused by over ventilation. This would reduce the whole effect to an expression of the Zuntz reaction, a shifting of the carbonates from the plasma to the cells in response to a lowering of the carbon dioxide concentration of the blood. If this were the case the alveolar air determination should reveal the effects of the "*Auspumpung*." The alveolar values, however, are higher than those obtained from the plasma, an indication that the respiratory mechanism has not been able to remove the increased CO_2 offered to it (3, 4). On the other hand, if we were dealing with a simple carbon dioxide acidosis the plasma carbonates should rise instead of fall. The dyspnea and the fall in plasma carbonates must, therefore, represent a real diminution of the fixed alkali of the blood.

The mechanism of epinephrin hyperglycemia and glycosuria, although not yet clear in all its details, can be ascribed most probably to an increase in glycogenolysis. The evidence has been thoroughly discussed in a recent paper by Mackenzie (5) and need be considered here only as it has a bearing on our special problem. The effects of epinephrin, especially as regards carbohydrate metabolism, are strikingly like those of acid though very little attention has been paid hitherto to this aspect of its behavior.

Elias (2) was the first to point out that the administration of acid produced a hyperglycemia and glycosuria and that this was due to an increased glycogenolysis. This action was independent of the adrenals and could be demonstrated even in the perfused liver washed free from blood. Alkali produced an opposite effect. He used hydrochloric acid and sodium carbonate. Macleod (6) found that the hyperglycemia and glycosuria of asphyxia were dependent on a carbon dioxide acidosis. Both the hyperglycemia and glycosuria failed to appear when the liver was excluded from the circulation, but were not prevented when the hepatic nerve plexus was cut. The parallelism between the action of acid and the action of adrenalin fails only inasmuch as the investigation on the effect of acid is not so complete as the investigation on the effect of adrenalin.

Underhill (1) was able to increase epinephrin hyperglycemia and glycosuria by the administration of hydrochloric acid, and, what is more significant, he was able to diminish hyperglycemia and glycosuria and even, in one case, to prevent it by the administration of sodium carbonate. The inhibitory effect of carbonate in preventing hyperglycemia and glycosuria was obtained only when it was injected at least half an hour before the epinephrin. This suggests that carbonate acts by establishing conditions unfavorable to the production of the regular adrenalin effect.

In view of this experimental evidence and the time relations of the CO_2 and hyperglycemia curves, it seems more than probable that at least a part of the hyperglycemia and glycosuria following the injection of adrenalin was caused by a diminution of the alkalinity of the blood.

Diminished blood alkalinity as the probable cause of hyperglycemia is particularly suggested by a study of the time relation of the curves in the three diabetic cases (1, 2, and 3). In the charts of these cases it will be noticed that the apex of the blood CO_2 curve is attained from 1 hour to 1 hour and 40 minutes before the apex of the blood sugar curve is reached. This did not occur in the two normal individuals whose blood sugar and blood CO_2 curves reached their peak simultaneously. A possible explanation of this discrepancy lies in the fact that in the diabetic the whole reaction extends over a longer period than in the normal cases. In this connection it is possible that determinations made sooner after the administration of adrenalin and at more frequent intervals in the normal cases would have shown a similar relationship.

We have recognized that the change in blood reaction and change in sugar content have occurred in all cases at the time of the first observation, that is, within 20 minutes. Whether it could be found that the changes occurred simultaneously and immediately after adrenalin was given or whether one precedes the other has not been shown.

Determinations at 2 to 5 minute intervals after adrenalin might possibly show that the blood CO_2 change preceded the blood sugar change, and if that were the case the evidence would be even more convincing that diminished alkalinity played the lead-

ing rôle in the production of hyperglycemia. It was difficult, however, to find patients willing to submit to such frequent vena puncture as this plan would necessitate and such estimations were not made.

The degree of acidemia which was produced by the injection of adrenalin was probably sufficient to bring about the increase in the blood sugar, for Elias by the injection of acids was able to induce glycosuria in dogs even when the acidemia was not sufficient to cause air hunger. In two of our cases the acidemia following adrenalin was accompanied by severe hyperpnea. That this hyperpnea was not out of proportion to the change of blood reaction was evident from the fact that it was not sufficient to lower the alveolar CO_2 tension to the level required by the concentration of carbonates in the plasma.

Ritzmann (7), who administered adrenalin intravenously, studied its parallel effect upon glycosuria and hypertension. He concluded that adrenalin affected carbohydrate metabolism only when it caused vasoconstriction. He did not make observations on the blood sugar.

Lusk (8) found that adrenalin was without influence upon the oxidation of sugar and agreed with Ritzmann that adrenalin acted upon carbohydrate metabolism by vasoconstrictor effect, the vasoconstriction causing asphyxia of the tissues. Pollak (9) showed that both hyperglycemia and glycosuria were more readily produced by subcutaneous than by intravenous administration of adrenalin. It is well known that vasoconstrictor effects are uncertain after subcutaneous administration. In two of our cases after the subcutaneous administration of adrenalin the rise in the blood sugar was accompanied by hypertension. In two others there was an increase in the blood sugar but no rise in the blood pressure. This does not rule out vasoconstriction of the liver vessels but a vasoconstriction sufficient to cause asphyxia of the tissues is at least improbable. Tissue asphyxia, however, in itself increases the acidity of the blood and tissues.

Epinephrin and acid also have other physiological properties in common. According to Trendelenburg (10) they both cause relaxation of the bronchial muscle and vasoconstriction. How far the parallelism can be carried and to what extent the action of adrenalin is dependent upon the associated acidosis it is impossible to say.

The discovery of an acid intoxication from adrenalin is strangely at variance with Crile's theory of shock, but the experimental evidence of an alkalinizing action on the part of the adrenals brought forward by Menten and Crile (11) is entirely unsatisfactory. Bedford (12) has recently shown that, contrary to Crile's statement, there is an increase in the adrenalin content of the blood flowing from the adrenals, during prolonged shock in dogs.

CONCLUSIONS.

1. The hyperglycemia produced by subcutaneous injection of adrenalin in three cases of diabetes and two normal individuals was accompanied by simultaneous diminution of the alkalinity of the blood. This taken in conjunction with other experimental evidence strongly suggests that decreased alkalinity of the blood plays a very important rôle in the production of hyperglycemia of this type.

2. Vasoconstriction as demonstrated by peripheral hypertension is not of prime importance in producing the changes noted.

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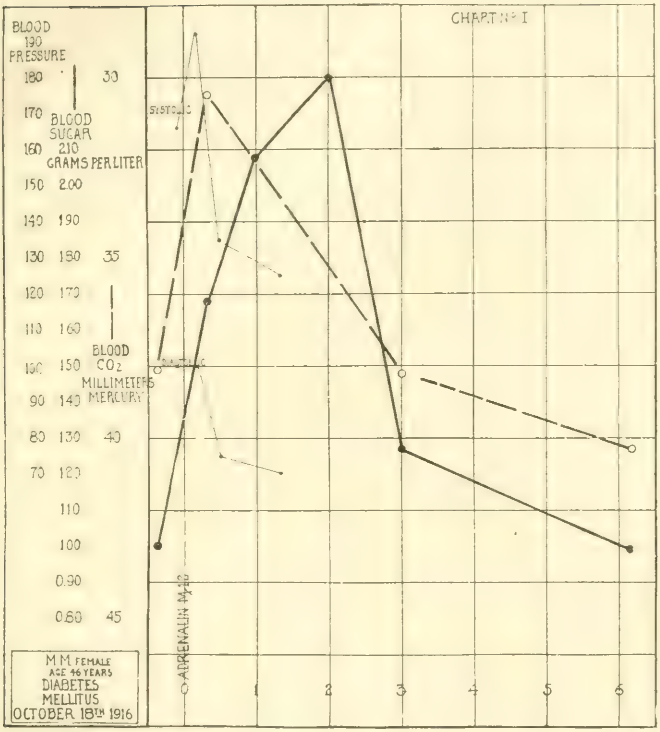
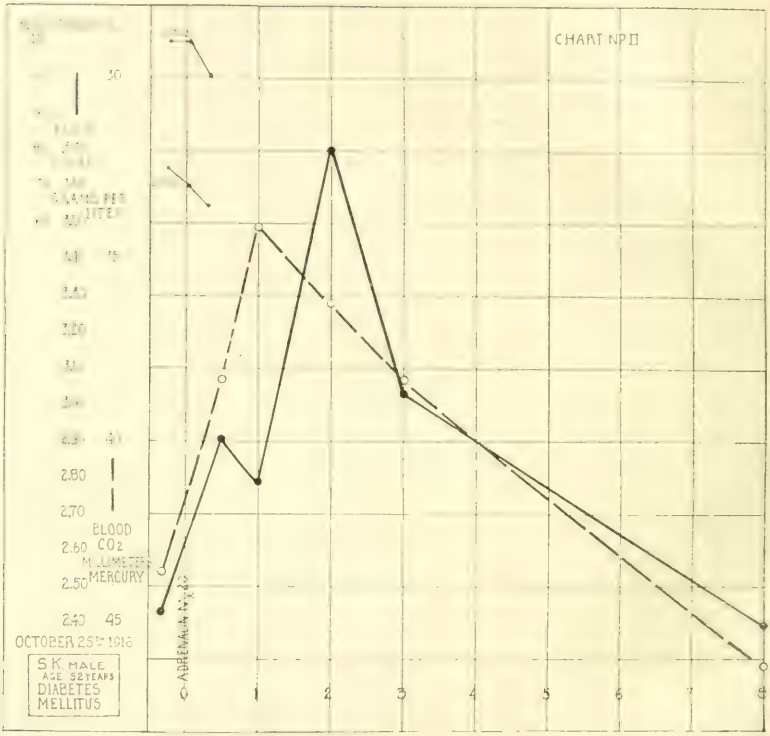


Case 1.—M. M. Female. Age 46 years. Diabetes mellitus of 2 to 3 years' duration.

Oct. 18, 1916, after a fast of 39 hours, adrenalin ♏12 injected subcutaneously at 9.30 a.m. Within a few minutes of its administration the patient complained of throbbing in head and throughout body; slight headache. Fibrillary twitching in muscles of arms and trunk; slight hyperpnea. Within 15 minutes after the administration of the drug all subjective symptoms had disappeared.

Pulse increased from 88 to 122, 7 minutes after adrenalin, and then dropped to 90.

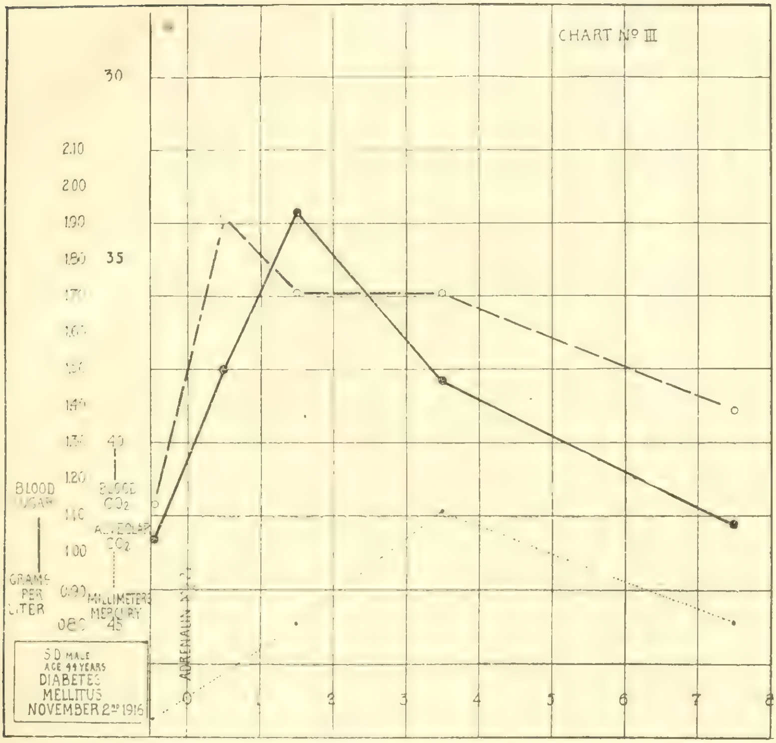
Urine.—Before adrenalin: sugar 0. After adrenalin: sugar 0.



Case 2.—S. K. Male. Age 52 years. Diabetes mellitus of 10 years' duration, and pulmonary tuberculosis of at least 2 years' duration.

Oct. 25, 1916, after a fast of 41 hours, adrenalin ♏20 given subcutaneously at 11.30 a.m. There were no subjective or objective symptoms observed. No increase in pulse rate.

Urine.—Before adrenalin: sugar 0. After adrenalin: taken hourly for 4 hours, contained 2.35 gm. of sugar. After this became sugar-free.

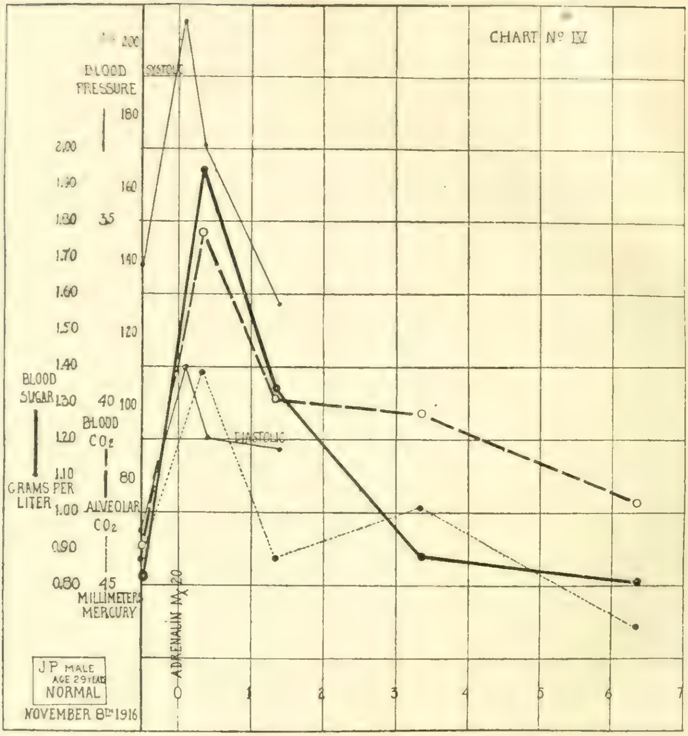


Case 3.—S. D. Male. Age 44 years. Diabetes mellitus of 5 years' duration.

Nov. 2, 1916, after a fast of 23 hours, adrenalin ℳ21 injected subcutaneously at 9 a.m. Almost immediately (within 5 minutes) the patient complained of throbbing throughout body, and felt as though "blood was all in legs and stomach." Hyperpnea quite marked. Complete subsidence of subjective symptoms within 10 minutes.

Pulse increased from 78 to 110, 5 minutes after adrenalin was given, and then dropped to 80 15 minutes after the adrenalin had been given.

Urine.—Before adrenalin: sugar 0. After adrenalin: 1st hour, sugar 0; 2nd hour, sugar faint trace; for remainder of the day, sugar 0.

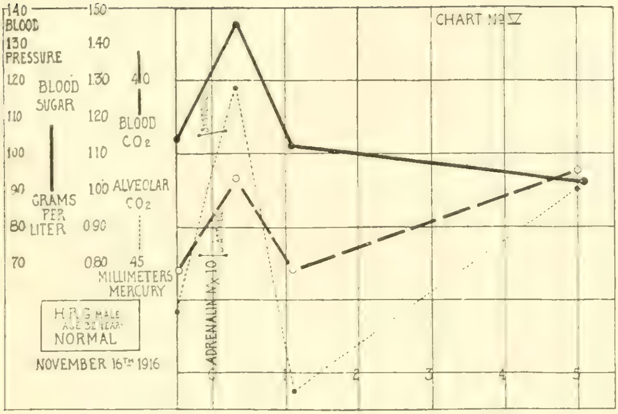


Case 4.—J. P. Male. Age 29 years. Normal.

Nov. 8, 1916, after a fast of 14 hours, adrenalin ℳ20 injected subcutaneously at 9.40 a.m. Within 3 minutes the patient became very pale and hyperpneic, with palpitation sensations and alternate numbness and tingling in hands and feet. Face showed anxiety and distress although he insisted that he enjoyed the sensations. 15 minutes later all symptoms had disappeared.

Pulse increased from 80 to 115, 5 minutes after adrenalin was given, then dropped to 90.

Urine.—Before adrenalin: sugar 0. After adrenalin: 1st hour, sugar trace; for subsequent 12 hours, sugar 0.



Case 5.—H. R. G. Male. Age 32 years. Normal.

Nov. 16, 1916, after fasting for 11 hours, adrenalin ♏10 given subcutaneously at 9.20 a.m. Within 5 minutes mild sensations of constriction of the head, which passed almost immediately. No hyperpnea.

Pulse rate not increased.

Urine.—Contained no sugar at any time.

A MODIFICATION OF THE McLEAN-VAN SLYKE METHOD FOR THE DETERMINATION OF CHLORIDES IN BLOOD.

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(Received for publication, July 19, 1917.)

The most satisfactory method for the determination of chlorides in blood is the one devised by McLean and Van Slyke.[1] This procedure involves removal of the blood proteins by heat, acetic acid, magnesium sulfate, and blood charcoal; precipitation of the chlorides from an aliquot part of the filtrate by means of a dilute standard silver nitrate solution containing nitric acid; removal of the precipitated silver chloride, and titration of the excess silver with dilute standard potassium iodide in the presence of nitrous acid and starch—the free nitric acid being, for the most part, neutralized by the addition of trisodium citrate. The end-point is extremely sensitive and the procedure gives accurate results with very small amounts of chloride.

This method, however, depends in part on the use of Merck's Blood Charcoal Reagent, which at the present time is not to be had. According to McLean and Van Slyke no other charcoal is satisfactory.

We have found that thoroughly reliable results are obtained by applying the original McLean-Van Slyke titration to the filtrates obtained after coagulating the blood proteins with metaphosphoric acid. The additional acidity due to the metaphosphoric acid is not sufficient to interfere with the sensitiveness of the starch-iodine end-point, being only about 2 per cent of that due to the nitric acid.[2] The coagulum formed by the addition of metaphosphoric acid to the diluted blood or plasma is very finely divided, and the diffusion of chlorides to uniform concentration through-

[1] McLean, F. C., and Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxi, 361.

[2] McLean and Van Slyke, *J. Am. Chem. Soc.*, 1915, xxxvii, 1128.

out the mixture is complete in 10 minutes if the flask is shaken occasionally. Our metaphosphoric acid, a J. T. Baker Chemical Company preparation, is free from chlorides.

The method, with the suggested modification, is described in full for the sake of completeness, though the operations after removing the blood proteins are simply those of McLean and Van Slyke, as also are the solutions used in the titration of the protein-free filtrate. We proceed as follows.

Take 2 cc. of plasma (or whole blood) in a 25 cc. volumetric flask. Add about 20 cc. of water and then slowly and with stirring add 1 cc. of a freshly prepared 25 per cent solution of metaphosphoric acid. Fill to the mark with water, shake well, and let stand for 10 minutes with occasional shaking. Filter and take 10 cc. of filtrate in a 25 cc. volumetric flask, add 5 cc. of the M/29.25 silver nitrate solution, 5 cc. of 10 per cent magnesium sulfate solution (to facilitate the flocking out of the silver chloride), fill to the mark with water, shake, and let stand for 5 minutes. Filter or centrifuge the liquid and take 20 cc. of the clear solution in a small Erlenmeyer flask, add 4 cc. of the nitrite, citrate, starch solution, and titrate with the M/58.5 potassium iodide solution.

The calculation is as follows: $1.25\ (8 - \text{cc. KI used}) \times \frac{5}{4} \times 100$, or, simplified, $156\ (8 - \text{cc. KI used})$ = mg. NaCl per 100 cc. of blood or plasma.

This modification makes the admirable micro titration method of McLean and Van Slyke still available, and the procedure for removing the blood proteins is, we think, somewhat simplified. Another point in its favor is that, if chloride determinations are to be done on whole blood it may be unnecessary in some cases to use a separate portion of blood, but the titration may be done on the filtrates obtained in the determination of non-protein nitrogen according to Folin and Denis,[3] or on the filtrates obtained in blood urea determinations according to a method soon to be published from this laboratory. In this case, since 5 cc. of blood are diluted to 50 cc. the above mentioned formula for the calculation does not hold true. If 10 cc. of a non-protein nitrogen or urea nitrogen filtrate were used, the formula would be:

$$1.25\ (8 - \text{cc. KI used}) \times 100\ \text{mg. NaCl per 100 cc. of blood.}$$

[3] Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 491.

Table I shows how perfectly the results by the modified procedure agree with those of the McLean-Van Slyke method.

TABLE I.

Sample.	Dilution.	Old method.		New method.	
		KI	NaCl per 100 cc.	KI	NaCl per 100 cc.
		cc.	mg.	cc.	mg.
Normal human plasma I	2:25	4.02	620	4.02	620
" " " I	2:25	4.04	618	4.00	623
" " " II	2:25	4.15	601	4.15	601
" " " II	2:25	4.15	601	4.17	598
Beef plasma III	2:25	4.22	590	4.20	594
" " III	2:25	4.24	587	4.24	587
" " III	2:25			4.22	590
" " III	5:50			3.25	595
" " III	5:50			3.27	592
Beef whole blood IV	2:25	4.80	500	4.78	503
" " " IV	2:25	4.76	505	4.80	500
" " " IV	5:50			3.98	502*
" " " IV	5:50			3.95	506*
" " " V	2:25	4.98	472	4.98	472
" " " V	2:25	5.00	469	4.96	474
" " " V	5:50			4.20	475**
" " " V	5:50			4.20	475**

* Non-protein nitrogen filtrates.

** Blood urea filtrates.

I am indebted to Professor Folin for his advice and encouragement in this work.

THE FUNCTION OF MUSCULAR TISSUE IN UREA FORMATION.

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(Received for publication, July 9, 1917.)

INTRODUCTION.

The formation of urea in the animal body is a question of fundamental importance in protein metabolism. Certain features of the problem have been worked out with some degree of satisfaction, but there is still uncertainty both as to where and how urea is formed in the body and as to the nature of the processes involved. The present status of our knowledge on the subject may be briefly summarized as follows.

The end-products of protein digestion—chiefly amino-acids—are absorbed directly into the circulatory system from the walls of the small intestines, and are then transported to the various body tissues. The body cells select such amino-acids as are needed for their repair or upbuilding, and quantities in excess of these needs are deaminized with the subsequent production of ammonium carbamate and ammonium carbonate, which are transformed into urea, and removed from the blood by the kidneys. The deaminized residue of the amino-acid molecule may then be utilized for the production of energy or it may be transformed into reserve material—glycogen or fat. In case of the break down of the protein constituents of the cell, it is probable that the amino-acids formed undergo changes similar to those which take place in the amino-acids absorbed from the digestive tract.

The important question, so far as the purposes of this paper are concerned, is: Where and by what means is urea formed in the body?

There is abundant proof that the liver performs an important function in urea formation. Satisfactory evidence that it is the sole source, or even the most important source, of urea production in the body is lacking. On the other hand, the prevailing theory is that urea formation is in all probability a function of body cells in general rather than of the liver cells in particular. It seems worth while to quote briefly the views of a few leading physiological chemists on this subject.

Otto von Fürth[1] states: "Im Ganzen neigt man gegenwärtig mehr und mehr der Meinung zu, dass die Fähigkeit, Harnstoff zu bilden, nicht ein Vorrecht der Leber, vielmehr, ebenso wie die Fähigkeit, Eiweiss zu verbrennen, eine der allgemeinen Eigenschaften lebender Zellen ist."

Abderhalden[2] says: "Aus dem vorliegenden Beobachtungen ergibt sich der Schluss, dass die Leber Harnstoff bildet. Der Versuch am überlebenden Organ hat das eindeutig bewiesen. Wahrscheinlich kann er auch von allen anderen Körperzellen erzeugt werden. Allerdings fielen bis jetzt die Versuche an anderen überlebenden Organen als der Leber negativ aus. Die Beobachtungen an Individuen mit pathologisch veränderter Leber und vor allem die Tierversuche, bei denen die Leber künstlich schwer geschädigt wurde, zwingen uns jedoch zu der Annahme, dass die verschiedensten Gewebe Harnstoff bilden können."

Folin[3] states: "The hypothesis that the urea forming process is thus probably largely a matter of muscle metabolism predicates of course nothing as to the nature of the process. In no way does it invalidate or weaken the prevailing view that deaminization rather than oxidation represents the first step in the formation of urea from amino-acid nitrogen. Ascribing to the muscles the greatest share in the urea formation represents, therefore, in no essential point a return to the earlier teachings of Pflüger or of Voit."

Bayliss[4] has this to say concerning the formation of urea in the body: "In the second place, certain experiments tend to show that the liver has not much more power of deamination than the other cells of the organism. The experiments of Lang (1904) and of Miss Bostock (1911) have shown that tissues *in vitro* are capable of deaminating amino-acids to some extent, but that the process is not quite the same as that in the living organism, since

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[1] Von Fürth, O., Probleme der physiologischen und pathologischen Chemie, Leipsic, 1913, ii, 101.

[2] Abderhalden, E., Lehrbuch der physiologischen Chemie, Berlin, 3rd edition, 1914, i, 586.

[3] Folin, O., and Denis, W., Protein metabolism from the standpoint of blood and tissue analysis. Third paper. Further absorption experiments with especial reference to the behavior of creatine and creatinine and to the formation of urea, *J. Biol. Chem.*, 1912, xii, 161.

[4] Bayliss, W. M., Principles of General Physiology, London, 1915, 265.

amides are more readily acted on *in vitro* than are amino-acids, while the contrary is the case in the organism.

"We may conclude that amino-acids are supplied to the tissues, and, with the exception of that small part used for repair or growth, are deaminated there.

"The great activity of the liver in the conversion of ammonia to urea makes it probable that the main part of the ammonia from the tissues is converted into urea in that organ."

The basis for the theory that urea formation is a function of body cells in general rather than of liver cells in particular is, of course, founded upon the idea that the metabolism of the individual cell is complete in itself. Thus, in case of muscle cells, it is well known that they contain several proteolytic enzymes which actively break down the cell proteins with the production of amino-acids and ammonia, if not urea. Hoagland, McBryde, and Powick[5] found that when muscular tissue of an ox was incubated for 100 days under aseptic conditions at 37°C. the amino-acid content of the tissue increased 741 per cent and the ammonia content 519 per cent. While the two constituents increased at somewhat nearly the same rate, yet at the end of the period the amino-acid nitrogen constituted 18.9 per cent of the total nitrogen of the tissue, while the ammonia nitrogen constituted only 1.6 per cent. Unfortunately no study was made of the urea content of the muscular tissue in those experiments. The results of the experiments indicate clearly that muscle cells have to a high degree the ability to break down their protein constituents into amino-acids, and to a lesser degree, into ammonia. The lower production of ammonia was probably due to certain limiting factors which are discussed by the authors of the above paper. These changes are in harmony with the theory that ammonia is an intermediate product in the transformation of amino-acids into urea. Now since muscle cells break down their protein constituents into amino-acids and ammonia, it is reasonable to expect that those cells might transform the ammonia into urea. At any rate, that is the idea which led the writers to undertake the investigations which are to be reported in this paper. Again, it may be that the change is carried no further than ammonia by the muscle cells, and perhaps by other

[5] Hoagland, R., McBryde, C. N., and Powick, W. C., Changes in fresh beef during cold storage above freezing, *U. S. Dept. Agric., Bull. 433*, 1917.

body cells as well, and that the ammonia is carried to the liver where it is transformed into urea. The well known marked ability of the liver to change ammonia into urea harmonizes with this view.

Direct experimental evidence that urea is formed elsewhere in the body than in the liver is comparatively meager. The problem is a difficult one to solve. It necessitates either the complete removal of the liver from the circulation of an animal and a study of urea production in the body under such conditions, the difficulties of such a procedure being obvious; or it requires the identification of urea-forming enzymes in body tissues other than the liver. There is considerable indirect evidence that urea may be formed elsewhere in the body than in the liver, but the value of such evidence is uncertain, and it will not be presented.

The following direct experimental evidence supports the view that urea formation is a function of body cells in general.

Matthews and Nelson[6] conducted experiments in which solutions of amino-acids were injected, in one case into the muscular tissue of dogs with Eck fistulas, and in the second case into dogs from which all the abdominal viscera except the kidneys and a small portion of the liver had been removed. In the first instance there was a slight increase in the ammonia and urea content of the urine after 2 to $2\frac{1}{2}$ hours. In the second instance there was always an increase in the ammonia content of the urine at the end of the 1st hour. The increase in the urea, which generally appeared from 1 to 2 hours later, was not so constant, only being manifest in about 90 per cent of the experiments. In some instances the only result obtained was a marked increase in ammonia accompanied by a decrease in urea.

Fiske and Sumner[7] injected solutions of amino-acids into animals in which the liver and kidneys had been removed from the circulation, and as checks, ran controls in which only the kidneys were removed from the circulation. The experimental period amounted to 3 hours or less. On the whole, it was found that there was an appreciable accumulation of urea both in the blood and the muscular tissue after the injection of amino-acids, and that the accumulation was practically as great when the liver was excluded from the circulation as when it was in its normal relations to the other organs. As a result of their experiments the authors conclude that the liver is not the chief site of urea formation from amino-acids.

With the exception of arginase, urea-forming enzymes have not been identified in body tissues.

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[6] Matthews, S. A., and Nelson, C. F., Metabolic changes in muscular tissue. I. The fate of amino-acid mixtures, *J. Biol. Chem.*, 1914, xix, 229.

[7] Fiske, C. H., and Sumner, J. B., The importance of the liver in urea formation, *J. Biol. Chem.*, 1914, xviii, 285.

EXPERIMENTAL.

The purpose of the experiments which are to be reported in this paper was to determine whether or not urea-forming enzymes are present in muscular tissue.

The general plan of the experiments was to obtain sterile samples of muscular tissue by aseptic methods and incubate the tissue in sterile containers for various periods of time, urea being determined in the fresh tissue and in the incubated samples. The general method of procedure was as follows.

Methods.

Fat steers were slaughtered at a local abattoir by the customary methods under the direction of the authors. The operation was carried on with as great dispatch and under as clean conditions as possible. Prior to slaughter the killing floor was washed with hot water and then with a solution of mercuric chloride (1: 1,000). The animal was stunned, hoisted from the floor, and the large blood vessels of the throat were severed. When bleeding was completed the carcass was dropped on to the clean killing floor and wet down with hot water. The proposed lines of incision for the removal of the hide were first scrubbed with hot water and soap and then with a hot solution of mercuric chloride (1: 1,000). The knives and saws used during slaughter were immersed in hot water and then placed in a hot solution of mercuric chloride from time to time during slaughter. One hind quarter from each animal slaughtered was selected for experimental purposes. The hide was removed from this quarter with as great care as possible to avoid contamination of the freshly exposed tissues. During the removal of the hide, cheese-cloth that had previously been soaked in mercuric chloride solution was wrapped around the surface of the hind quarter as rapidly as the hide was removed; and finally when it had been separated from the carcass, the quarter of beef was wrapped with dry cheese-cloth and paper and immediately and rapidly transported to the laboratory. At the laboratory the outer coverings were removed from the quarter of beef, and it was placed in a special room where samples of the muscular tissue were taken for incubation and analysis.

Method of Taking Samples.

The room in which the samples of muscular tissue were taken was about 10 ft. square and provided with a false cheese-cloth ceiling at a height of 10 ft. A window which opened into the room was fitted with a cheese-cloth screen. Prior to taking samples the room was thoroughly washed and then sprayed with a 3 per cent solution of liquor cresolis compositus.

The operators wore sterile gowns, head cloths, and rubber gloves while taking the samples.

The bichloride cheese-cloth was first removed from the quarter of beef and the exposed surface was then wet down with a solution of bichloride of mercury and wiped dry with a sterile cloth. The proposed lines of incision were sterilized by means of a large steel spatula previously heated to a bright red color. Sterile knives and forceps were used in taking all samples. The seared lines were first cut through to a depth of about 0.5 cm. and a second knife was used to trim back sufficient surface tissue to expose the area of muscular tissue necessary in order to get a sample of the desired size. A third knife was used to cut out a block of muscular tissue, which was immediately taken up by means of a pair of forceps and placed in a sterile glass covered dish; additional samples were taken in a similar manner. Every possible precaution was taken to prevent contamination of the samples taken for incubation.

The dishes in which the samples were incubated are 10 cm. high and 10 cm. in diameter and are provided with loosely fitting glass covers. They are of the type known as "dressing jars." As soon as the desired number of samples had been obtained the dishes were sealed by means of strips of adhesive tape, which were then painted with melted paraffin so as to make the seal practically air-tight. The dishes were weighed empty, after the introduction of the samples of tissue, and at the end of the incubation periods. The dishes were placed in an incubator and held there at 37°C. for various periods of time.

The samples of muscular tissue taken for study were secured from the following muscles: biceps femoris, semimembranosus, semitendinosus, and vastus externus.

Bacteriological Examination of Samples.

The incubated samples were examined from time to time and those which showed apparent evidences of bacterial contamination were discarded. Samples which appeared to be sterile were selected at intervals and subjected to a careful bacteriological examination to determine whether bacteria were present or not. Chemical results are reported only on samples proven to be free from bacteria. The following procedure was employed in the bacteriological examination of the samples.

The tape was first removed from the jar and the edges of the cover were flamed. Since considerable quantities of juice had exuded from the samples of muscular tissue during incubation, the juice as well as the tissue was examined for the presence of bacteria. 0.5, 0.25, and 0.1 cc. portions of the juice were plated with standard agar. Portions of tissue were first taken from the surface of the sample, it was then cut in two, and similar cultures were taken from the interior, sterile knives and forceps being used. Three portions were taken from the surface and a like number from the interior. One portion from each surface was placed in peptonized beef

broth for the development of aerobic bacteria and two portions were introduced into tubes of glucose agar for the development of anaerobic organisms. The tubes of agar had first been boiled to drive off traces of air and then allowed to cool to approximately 40°C. The portion of tissue was introduced into the tube and as soon as the material had settled to the bottom the tube was placed in ice water for the agar to harden so as to exclude air and encourage the growth of anaerobic organisms. Smears were also made on cover glasses and examined for bacteria. All cultures were incubated for at least 6 days, and in the absence of growth the samples of muscular tissue were accepted as being sterile.

Methods of Analysis.

The muscular tissue was freed as far as practicable from visible fat and connective tissue, and finely ground in a meat grinder. In case of the incubated samples, from which considerable juice had exuded, the tissue was first ground and then thoroughly mixed with the juice. Analytical work was always started immediately after the sample had been prepared for analysis.

Urea was determined by the well known urease method. Duplicate determinations were made on all samples, and the average result is reported. The procedure employed was as follows.

25 gm. of the ground tissue were introduced into a 200 cc. Erlenmeyer flask, and 100 cc. of absolute alcohol added. The flask was stoppered and shaken to break up lumps of tissue and the shaking was repeated at intervals during an extraction period of about 20 hours. The alcoholic extract was then decanted into a beaker and the tissue residue was transferred to a mortar and ground in the presence of sand. The ground tissue and alcoholic extract were returned to the flask and extraction was continued for 2 to 3 hours or longer with occasional shakings. The contents of the flask were then filtered on an asbestos filter with the aid of suction, and the flask and filter were washed with hot 95 per cent alcohol. The filtrate was transferred to a beaker and evaporated to about 10 cc. on a steam bath, but never to dryness. The contents of the beaker were transferred to a 25 cc. volumetric flask and made to volume.

A 10 per cent solution of a dry commercial preparation of urease containing the proper quantity of mixed phosphates to insure the maximum activity of the enzyme was made up just before each set of determinations was to be made.

The apparatus used for the determination of urea and ammonia in the extracts of the tissue is of the same general character as that commonly used for the determination of ammonia by the Folin aeration method, and it need not be described in detail. Test-tubes $6\frac{1}{2}$ inches long and $1\frac{1}{8}$ inches

in diameter and having a capacity of about 100 cc. were used to hold the tissue extract.

25 cc. of N/50 sulfuric acid, three drops of octyl alcohol, 7 drops of a 0.05 per cent solution of methyl red, and 50 cc. of water were introduced into each absorption cylinder. 10 cc. of the concentrated tissue extract were introduced into each of two 100 cc. test-tubes, one portion for the determination of ammonia, the other for urea and ammonia combined. To the tube containing the first portion were added 15 cc. of water and 7 drops of octyl alcohol, and to the second tube were added 2 cc. of a 10 per cent solution of urease, 7 drops of octyl alcohol, and 13 cc. of water. The tubes were connected with the apparatus and allowed to stand 45 minutes for the urease to convert the urea into ammonia, when air was drawn through the apparatus at a moderate rate for 3 to 5 minutes. Suction was then turned off, the test-tubes were disconnected, and 12 gm. of potassium carbonate were added to each tube containing tissue extract. The tubes were immediately connected with the apparatus and air was drawn through it, slowly at first, then as rapidly as practicable, for a total period of at least an hour. The excess of acid in the absorption cylinders was titrated against N/50 NaOH.

In making calculations correction was made for the ammonia liberated from the aliquot portion of the tissue extract which had not been treated with urease. The ammonia liberated from urea by the action of urease was calculated in terms of percentages of urea in the original tissue. In cases where the samples of muscular tissue had lost weight during incubation, the data have been corrected for that loss. Following the procedure which has been described it was found that when known quantities of urea were added to samples of muscular tissue the amount present could be determined with a high degree of accuracy.

Experiment 1.—A fat steer was slaughtered at a local abattoir and one hind quarter was transported to the laboratory, the procedure requiring 1 hour and 25 minutes. Samples were taken for incubation from the following muscles: biceps femoris 11, semimembranosus 9, vastus externus 7, and semitendinosus 3, total 30. The weights of the samples ranged from 90 to 409 gm., the average weight being 226 gm. Such large samples were taken because a study was being made not only of possible urea-forming enzymes but of other muscle enzymes as well. Samples were also taken from each muscle for the determination of urea in the fresh tissue. The samples taken for incubation were placed in an incubator at 37°C. 8 hours and 20 minutes after the death of the animal. The analyses of the fresh tissue were started 7½ hours after the animal was killed. The incubated samples were examined macroscopically at the end of 24, 48, and 72 hours, and subsequently at less frequent inter-

vals. Samples showing positive evidences of contamination were discarded. Usually, though not always, samples which showed no macroscopic evidence of bacterial contamination after incubation for 7 days would prove to be sterile upon careful bacteriological examination. A total of 7 sterile samples, or 23.3 per cent of the samples taken, was obtained from this quarter of beef. While this seems like a rather small percentage of sterile samples, yet every possible precaution was taken to avoid contamination of the samples taken. The difficulties attending the operation of securing sterile samples of muscular tissue weighing several hundred gm. by aseptic methods can be appreciated only by one who has undertaken such an experiment. The changes in the urea content of the muscular tissue during incubation for various periods of time are shown in Table I. "Incubation period" as used in this and following tables denotes the time which elapsed between the death of the animal and the starting of the analysis.

TABLE I.

Serial No.	Samples.	Incubation period.		Urea.
		days	*hrs.*	*per cent*
7	Biceps femoris.		$7\frac{1}{2}$	0.0180
11	" "	2	3	0.0201
12	" "	3	4	0.0226
13	" "	8	2	0.0241
14	" "	15	2	0.0206
15	" "	22	3	0.0262
8	Vastus externus.		$7\frac{1}{2}$	0.0176
16	" "	29	3	0.0172

Experiment 2.—In this experiment samples of tissue were taken from the biceps femoris and semimembranosus muscle, in this instance about 30 minutes after the death of the animal, and the urea determinations were started at the abattoir. The analyses were started about 70 minutes after the animal was killed. At the laboratory additional samples were taken from the above named muscles for incubation. 16 samples were taken from the biceps femoris and 6 from the semimembranosus muscles, a total of 22. The weights of the samples ranged from 124 to 323 gm., the average weight being 224 gm. The samples were incubated at 37°C. for periods ranging from $5\frac{1}{2}$ hours to 42 days. Of these samples, 7 from the biceps femoris muscle were found to be sterile, or 31.8 per cent of the total number of samples taken. On account of lack of material for all the determinations which were being made, urea was determined in only 4 of the 7 sterile samples. The urea contents of the fresh and incubated samples of muscular tissue are shown in Table II.

TABLE II.

Serial No.	Samples.	Incubation period.		Urea.
				per cent
18	Biceps femoris.	1 hr.	10 min.	0.0165
23	" "	3 days.	2 hrs.	0.0153
28	" "	27 "	1 hr.	0.0180
39	" "	34 "	3 hrs.	0.0178
41	" "	41 "	3 "	0.0153

Experiment 3.—In this experiment also the urea determinations were started at the abattoir. At the laboratory additional samples were taken from the biceps femoris and semimembranosus muscles for analysis and incubation. With one exception, samples of tissue were taken for analyses at 2 hour intervals up to 12 hours from the time the animal was killed. Subsequently samples were analyzed at less frequent intervals. Samples of tissue that were selected for analysis 2 and 4 hours after slaughter were taken direct from the quarter of beef and were not subjected to bacteriological examination, since there had been no opportunity for bacterial penetration of the tissue. A total of 13 samples was taken for incubation, 6 from the biceps femoris and 7 from the semimembranosus muscles. Six of the samples proved to be sterile, or 46 per cent of the total number of samples incubated. Urea determinations were made on only 4 of the 6 sterile incubated samples. The urea contents of the fresh and incubated samples of muscular tissue are shown in Table III.

TABLE III.

Serial No.	Samples.	Incubation period.		Urea.
				per cent
29	Biceps femoris.	1 hr.		0.0156
31	" "	2 hrs.	45 min.	0.0156
32	" "	4 "	15 "	0.0161
33	" "	6 "	20 "	0.0155
35	" "	10 "	30 "	0.0159
36	" "	2 days.	3 hrs.	0.0142
29	Semimembranosus.	1 hr.		0.0161
46	"	20 days.	4 hrs.	0.0163

DISCUSSION OF RESULTS.

In Experiment 1, as shown by the data presented in Table I, there is apparently a slight increase in the urea content of the biceps femoris muscle on incubation for periods varying from 2

days and 3 hours to 22 days and 3 hours. The increases do not proceed at a regular rate, however, the sample incubated 15 days and 3 hours containing practically the same amount of urea as the sample incubated 2 days and 3 hours. The vastus externus muscle shows practically the same urea content after incubation for 29 days and 3 hours as that of the fresh tissue. On the whole, these data do not indicate that the samples of muscular tissue examined had any appreciable ability to form urea.

In Experiment 2, as indicated by the results shown in Table II, there were only slight changes in the urea content of samples of the biceps femoris muscle incubated for periods ranging from 3 days and 2 hours to 41 days and 3 hours. These changes are irregular in character and are probably due to experimental error. These data do not indicate that the muscular tissue examined had any ability to form urea.

The data presented in Table III, Experiment 3, show practically no change in the urea content of samples of the biceps femoris muscle incubated for periods ranging from 2 hours and 45 minutes to 2 days and 3 hours. The slight changes are practically within the limit of experimental error. These data are of particular interest in showing practically no change in the urea content of the muscle either prior to rigor mortis or subsequently. There was no change in the urea content of the semimembranosus muscle after incubation for 20 days and 4 hours. The data presented in this table indicate that the muscular tissue examined lacked the property of converting its protein constituents into urea.

On considering the results of the experiments as a whole, it may be well to point out the extent to which the precursors of urea, amino-acids and ammonia, accumulate in muscular tissue during aseptic autolysis. These constituents were not determined in the experiments here reported, since Hoagland, McBryde, and Powick[5] had previousy reported a study of the changes in the amino-acid nitrogen and ammoniacal nitrogen content of muscular tissue during aseptic autolysis. Table IV has been prepared from data presented in the report by the above authors and it indicates not only the changes in the ammoniacal nitrogen and amino-acid nitrogen content of the muscular tissue during autolysis, but also the possible urea value of the sum of the two constituents.

TABLE IV.

Composition Expressed in Terms of Percentages of Fresh Material.

Serial No.	Incubation period.	Ammoniacal nitrogen.	Amino-acid nitrogen.	Possible urea.
	days			
109	1	0.0087	0.0782	0.1867
110	8	0.0185	0.1412	0.3430
111	15	0.0225	0.1931	0.4631
112	22	0.0254	0.2101	0.5059
113	29	0.0349	0.4232	0.9840
120	43	0.0365	0.4641	1.0750
121	65	0.0509	0.4715	1.1230
122	78	0.0476	0.6100	1.4125
124	94	0.0588	0.6208	1.4598
125	101	0.0629	0.7658	1.7800

The results presented in Table IV are of interest in showing the marked accumulation of the precursors of urea in muscular tissue autolyzed for various periods of time as compared with the urea content of other samples of muscular tissue autolyzed for similar periods, as shown in Tables I, II, and III. For example, in Table II the biceps femoris muscle contained 0.0165 per cent urea 70 minutes after the death of the animal, while a sample of the same muscle incubated for 41 days at 37°C. contained 0.0153 per cent urea, indicating practically no change in the urea content of the tissue. On the other hand, by referring to Table IV it will be seen that the sample of muscular tissue that had been incubated for 43 days contained 1.075 per cent of the precursors of urea expressed in terms of that compound. Thus in case of the second sample the amino-acid and ammoniacal nitrogen, expressed in terms of urea, amounted to 70 times the actual urea content of the sample incubated 41 days. Without going into a detailed discussion of the relations between the amino-acid and ammoniacal nitrogen content of the autolyzed samples of muscular tissue as shown in Table IV, and the urea content of other samples of tissue autolyzed for similar periods, as indicated in Tables I, II, and III, the fact that there is a marked increase in the precursors of urea in the muscular tissue, but that there is practically no change in the actual urea content of the tissue, indicates that the muscular tissue, of itself, lacks the ability to form urea. The proof is not absolute, of course, but it is highly presumptive.

In this connection it should be mentioned that the enzyme arginase discovered by Kossel and Dakin[8] cannot be regarded as an important urea-forming agent in muscular tissue since the above authors reported the enzyme to be present only in traces in that tissue, while Mathews[9] states that arginase is absent from muscular tissue.

SUMMARY.

Taken as a whole, the results of the experiments reported in this paper tend to show that urea formation is not an important function of muscular tissue. It must be admitted that these findings are contrary to what was expected, and to the more or less generally accepted theory that urea formation is a function of body cells in general; but no other interpretation of the facts seems possible.

In view of the well established fact that the liver plays an important part in the formation of urea in the body, these findings lend support to the view that urea production is chiefly a function of that organ.

[8] Kossel, A., and Dakin, H. D., Weitere Untersuchungen über fermentative Harnstoffbildung, *Z. physiol. Chem.*, 1904, xlii, 184.

[9] Mathews, A. P., Physiological Chemistry, New York, 2nd edition, 1916, 669.

GLYCOLYTIC PROPERTIES OF MUSCULAR TISSUE.

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INTRODUCTION.

Our knowledge concerning the processes by which carbohydrates are utilized by the animal body is very unsatisfactory. It may be safely assumed, however, that the entire cycle of changes which sugars undergo after absorption into the circulatory system of the body is due to the action of enzymes; but experimental evidence in support of this assumption is very incomplete. Since the discovery of zymase by Buchner (1) there is no good reason for thinking that the sugar-oxidizing properties of the body are intimately bound up with the protoplasm of the living cell, any more than that alcoholic fermentation is dependent upon the living yeast plant.

A large amount of experimental work has been done in an attempt to identify glycolytic enzymes in muscular and other body tissues, and in the blood as well. The question as to whether the pancreas secretes a coenzyme or activator necessary for the activity of the glycolytic enzyme of the other body tissues has also been the subject of a great deal of study. Unfortunately the present state of our knowledge concerning the subject is very much confused. A considerable amount of work has been done which apparently shows that muscular and certain other body tissues, entirely apart from any secretion of the pancreas, have the property of oxidizing sugars. On the other hand, a considerable number of workers report results which apparently indicate that muscular and other body tissues, either singly or in combination with extracts from the pancreas, have no glycolytic properties whatever. A third group of workers claims that muscular tissue of itself has but very slight or no glycolytic properties, but that in the

presence of an activator supplied by the pancreas glycolysis takes place. The evidence is so conflicting and confusing that it is useless to discuss the work which has been done in any detail in an attempt to arrive at the truth of the matter. It does seem worth while, however, to present the names of the more important investigators of this subject.

The most ardent supporter of the theory that muscular and other body tissues contain enzymes which, apart from any coenzyme or activator supplied by the pancreas, oxidize sugar is Stoklasa (2–6), who has contributed a large amount of apparently very painstaking work on the subject. His work has been confirmed, in part at least, by that of Blumenthal (7), Brunton and Rhodes (8), Feinschmidt (9), Nanking (10), and Ransom (11).

Cohnheim (12–15), on the other hand, in his earlier work held that muscular and other body tissues had but very slight or no glycolytic properties of themselves, but that in the presence of an activator supplied by the pancreas glycolysis takes place. In his later work he modifies his earlier views somewhat by stating that body tissues are not always without glycolytic properties, but that these properties vary greatly. He still insists that the glycolytic properties of the tissues are greatly stimulated by the addition of an extract of the pancreas. His work is supported by that of Hirsch (16), Arnheim and Rosenbaum (17), Sehrt (18), and Hall (19).

Claus and Embden (20, 21) were unable to confirm the results obtained by Cohnheim, and found that muscular tissue of itself had practically no glycolytic properties. Similar results were obtained by McGuigan (22), Simpson (23), Harden and MacLean (24), and Levene and Meyer (25).

The conflicting nature of our knowledge concerning the behavior of sugars in the animal body simply emphasizes the importance of carrying on investigations in such a manner as will furnish exact and incontrovertible evidence on the problem. Clearly the most important point, and the one which should be established first, is: Do muscular and other body tissues of normal animals possess active glycolytic properties, entirely apart from the action of the secretion of any internal organ? That is the problem with which this paper is concerned.

EXPERIMENTAL.

I.

The general plan of the work was to secure sterile samples of muscular tissue from cattle immediately after slaughter, using aseptic methods, and to incubate the pieces of tissue in sterile

containers for various periods of time. The glycogen and the dextrose contents of the tissue were determined as soon as possible after the death of the animal and at intervals thereafter. A study was also made to determine the nature of the end-products of the changes in the carbohydrates of the tissue.

Method of Procedure.

The methods employed in the slaughter of the animals, the taking of samples of tissue for incubation, and the bacteriological examination of the incubated samples are essentially the same as those described in a previous paper (26) by the authors.

Methods of Analysis.

The muscular tissue was freed as far as practicable from visible fat and connective tissue and finely ground in a meat grinder. In case of the incubated samples, from which considerable juice had exuded, the tissue was first ground and then thoroughly mixed with the juice. Analyses were always started immediately after the preparation of the samples.

Glycogen was determined essentially according to the method of Pflüger as described by Abderhalden (27). The purified glycogen was inverted and dextrose was determined by means of Fehling's solution, reduced copper being estimated by Low's copper iodide method. Single determinations were run in the separation of the glycogen from the tissue, but duplicate estimations were made of dextrose in the inverted glycogen solution. Average results are reported in terms of dextrose.

Dextrose was determined by a method previously described by Hoagland (28). Experience has proven the method to yield accurate results. The possibility that a part of the glycogen might be converted into dextrose during the extraction of the sugar from the tissue and the subsequent concentration of the extract was recognized. In order to guard against such a change an excess of calcium carbonate was added to the tissue on extraction, and also to the extract on concentration. The concentrated extract was filtered from the lime residue which was washed free of sugar. In order to determine whether this procedure prevented any conversion of glycogen into dextrose, known quantities of glyco-

gen were added to like portions of a sample of muscular tissue, while no glycogen was added to a similar portion of the same sample, and dextrose was determined in each portion. It was found that the presence of glycogen was without effect upon the dextrose content of the samples examined.

That there might be an appreciable variation in the carbohydrate content of different parts of the same muscle was recognized as another possible source of error. In order to secure information on this point the biceps femoris muscle was dissected out from a hind quarter of beef and freed from surface fat and connective tissue. The muscle weighed 7,420 gm. Sugar was determined in five samples of muscular tissue taken from various parts of the muscle with the following results: 0.228, 0.256, 0.264, 0.255, 0.220 per cent, averaging 0.245 per cent.

From these data it will be seen that the maximum variation amounts to 0.044 per cent, while the greatest variation from the average is 0.025 per cent.

Experiment 1.—A fat steer was slaughtered at a local abattoir, and one hind quarter was transported to the laboratory, the procedure requiring 1 hour and 25 minutes. Samples of tissue were taken from the following muscles: biceps femoris 11, semimembranosus 9, vastus externus 7, and semitendinosus 3, a total of 30. The weights of the samples varied from 90 to 409 gm., the average weight being 226 gm. Portions were taken from each muscle for the determination of glycogen and dextrose in the fresh tissue. The samples taken for incubation were placed in an incubator at 37°C. 8 hours and 20 minutes after the death of the animal. The analyses of the fresh tissue were started 7½ hours after the animal was killed. The incubated samples were examined macroscopically at the end of 24, 48, and 72 hours and subsequently at less frequent intervals. Samples showing positive evidences of contamination were discarded, while those which appeared to be sterile were subjected to careful bacteriological examination. In this and subsequent experiments, unless otherwise stated, analyses are reported only on those incubated samples which were found to be sterile. A total of 7 sterile samples, or 23.3 per cent of the number of samples incubated, was obtained from this quarter of beef. The changes in the glycogen, dextrose, and total carbohydrate content of the samples of muscular tissue incubated for various periods of time are shown in Table I.

Experiment 2.—In this experiment samples of tissue were taken about 30 minutes after the death of the animal, from the biceps femoris and semimembranosus muscles, and glycogen and dextrose determinations were started at the abattoir about 70 minutes after the animal was killed. The quarter of beef was transported to the laboratory where additional samples were taken from the above named muscles for incubation and analysis.

TABLE I.*

Serial No.	Samples.	Incubation period.		Glycogen calculated as dextrose.	Dextrose.	Glycogen and dextrose calculated as dextrose.	Change in total carbohydrates.
		days	*hrs.*	*per cent*	*per cent*	*per cent*	*per cent*
7	Biceps femoris muscle.		$7\frac{1}{2}$	0.064	0.163	0.227	
11	" " "	2	3	Trace.	0.239	0.239	+5.3
12	" " "	3	4	"	0.180	0.180	−20.7
13	" " "	8	2		0.186	0.186	−18.1
14	" " "	15	2		0.179	0.179	−21.1
15	" " "	22	3		0.490	0.490	+116.0
8	Vastus externus "		$7\frac{1}{2}$	0.018	0.159	0.177	
16	" " "	29	3		0.263	0.263	+48.6
17	" " "	36	6		0.157	0.157	−11.3

* "Incubation period," as used in this and the three tables following, denotes the time which elapsed between the death of the animal and the starting of the analysis.

Sixteen samples were taken from the biceps femoris and 6 from the semimembranosus muscles, a total of 22. The weights of the samples varied from 124 to 323 gm., the average weight being 224 gm. The samples were placed in an incubator $5\frac{1}{2}$ hours after slaughter and were held there at 37°C. for periods ranging from $5\frac{1}{2}$ hours to 41 days. Seven samples from the biceps femoris muscle were found to be sterile after incubation, or 31.8 per cent of the number of samples incubated. Cultures were not taken from the sample analyzed $5\frac{1}{2}$ hours after the death of the animal since there had been no opportunity for bacterial invasion of the tissues. The glycogen, dextrose, and total carbohydrate content of the fresh and incubated samples are shown in Table II.

Experiment 3.—Samples of tissue were taken for analysis immediately after the death of the animal and at approximately 2 hour intervals up to 10 hours from the time the animal was killed, and subsequently at less frequent intervals. Samples taken for analysis 2 and 4 hours after slaughter were not subjected to bacteriological examination since there had been no opportunity for bacterial penetration of the tissues. A total of 13 samples was taken for incubation, 6 from the biceps femoris and 7 from the semimembranosus muscles. The samples were placed in an incubator at 37°C. $5\frac{1}{2}$ hours after slaughter. Six samples were found to be sterile after incubation, or 46 per cent of the number incubated. Three of the sterile samples, Nos. 36, 46, and 47, were incubated in an atmosphere of carbon dioxide. The glycogen, dextrose, and total carbohydrate content of the fresh and incubated samples of muscular tissue are shown in Table III.

TABLE II.

Serial No.	Samples.	Incubation period.	Glycogen calculated as dextrose.	Dextrose.	Glycogen and dextrose calculated as dextrose.	Change in total carbohydrates.
			per cent	per cent	per cent	per cent
18	Biceps femoris muscle.	70 min.	0.274	0.08	0.354	
20	" " "	$5\frac{1}{2}$ hrs.	0.014	0.051	0.065	−81.1
21	" " "	$10\frac{1}{2}$ "	0.017	0.132	0.149	−57.9
22	" " "	2 days. 1 "		0.158	0.158	−55.4
23	" " "	3 " 2 "		0.155	0.155	−56.2
28	" " "	27 " 1 "		0.030	0.030	−91.5
39	" " "	34 " 3 "		0.062	0.062	−82.5

TABLE III.

Serial No.	Samples.	Incubation period.	Glycogen calculated as dextrose.	Dextrose.	Glycogen and dextrose calculated as dextrose.	Change in total carbohydrates.
			per cent	per cent	per cent	per cent
29	Biceps femoris muscle.	1 hr.	0.672	0.132	0.804	
31	" " "	2 hrs. 45 min.	0.930	0.139	1.069	+33.0
32	" " "	4 " 15 "	0.843	0.133	0.976	+21.4
33	" " "	6 " 20 "	0.731	0.170	0.901	+12.0
34	" " "	8 " 30 "	0.417	0.294	0.711	−11.6
35	" " "	10 " 30 "	0.200	0.322	0.522	−35.0
36	" " "	27 "	0.267	0.447	0.714	−11.1
30	Semimembranosus "	1 "	1.015	0.138	1.153	
46	" "	20 days 4 hrs.		0.462	0.462	−60.0
47	" "	28 " 3 "		0.466	0.466	−59.6

Experiment 4.—Samples were taken for analysis as in Experiment 3. Those taken for analysis 3, $4\frac{1}{2}$, and $6\frac{1}{2}$ hours after slaughter were not subjected to bacteriological examination since there had been no opportunity for bacterial penetration of the tissues. Fourteen samples were taken for incubation, 7 from the biceps femoris and 7 from the semimembranosus muscles. The samples were placed in an incubator at 37°C. 6 hours after slaughter. Three of these samples, or 21.4 per cent of the number incubated, were found to be sterile. Cultures from one sample, No. 56, showed

no growth for 6 days after incubation, but later a slow-growing anaerobic organism developed. The analysis of this sample will be reported in Table IV since it is believed that there must have been but very slight development of the organism in the sample of muscular tissue during the short period of incubation to which it was subjected, a total of 8 hours and 20 minutes. The sterile samples were all obtained from the biceps femoris muscle. The changes in the glycogen, dextrose, and total carbohydrate content of the samples of muscular tissue are shown in Table IV.

TABLE IV.

Serial No.	Samples.	Incubation period.		Glycogen calculated as dextrose.	Dextrose.	Glycogen and dextrose calculated as dextrose.	Change in total carbohydrates.
				per cent	*per cent*	*per cent*	*per cent*
50	Biceps femoris muscle.	1 hr.	10 min.	0.734	0.170	0.904	
52	" " "	3 hrs.		0.564	0.171	0.735	−18.7
53	" " "	4 "	30 "	0.664	0.208	0.872	− 3.5
54	" " "	6 "	30 "	0.430	0.134	0.564	−37.6
*56	" " "	8 "	20 "	0.298	0.362	0.660	−27.0
57	" " "	10 "	30 "	0.148	0.437	0.585	−35.3
58	" " "	12 "	15 "	0.172	0.312	0.484	−46.4
61	" " "	2 days	1 hr.	0.097	0.464	0.561	−37.9
64	" " "	7 "	5 hrs.		0.379	0.363	−59.8
51	Semimembranosus "	1 hr.	10 min.	0.978	0.153	1.131	
55	" "	6 hrs.	30 "	0.257	0.386	0.643	−43.1

* Cultures show slow-growing anaerobic organism after 6 days.

DISCUSSION OF RESULTS.

The experiments will first be discussed separately, and then as a whole, in order to make clear the significance of the results obtained.

Experiment 1.—In this experiment the importance of determining the carbohydrate content of the muscles immediately after the death of the animal was not fully appreciated, so the first analysis was not started until 7½ hours after slaughter. It does not seem worth while to pay particular attention to the changes in the glycogen or the dextrose content of the muscles, but rather to the changes in the sum of the two constituents expressed in terms

of dextrose. These changes are shown most clearly in the column headed "change in total carbohydrates," where the changes are expressed in percentage of the amount of carbohydrates present in the first sample of each muscle analyzed.

The sample of biceps femoris muscle incubated 2 days and 3 hours shows an apparent increase in total carbohydrates amounting to 5.3 per cent, which is practically within the limit of experimental error, while the samples incubated for periods ranging from 3 days and 4 hours to 15 days and 2 hours show decreases in total carbohydrates amounting to approximately 20 per cent. The sample of this muscle incubated 22 days and 3 hours shows an increase in carbohydrates amounting to 116 per cent. The vastus externus muscle shows an increase of 48.6 per cent in total carbohydrates after incubation for 29 days and 3 hours, which confirms the increase in the carbohydrate content of the biceps femoris muscle. The sample incubated 36 days and 6 hours contains 11.3 per cent less carbohydrates than the first sample of this muscle analyzed.

Taken as a whole, the results of this experiment show (1) samples of tissue from both the muscles examined had appreciable glycolytic properties, the decreases in total carbohydrates varying from 11.3 to 21.1 per cent; (2) samples from both muscles apparently had the ability to synthesize carbohydrates.

Experiment 2.—The results obtained in the first experiment suggested the idea that in all probability the glycolytic properties of muscular tissue are most marked for a short time after death, and that these activities are brought to a close by the development of certain conditions associated with rigor mortis. For this reason samples of muscular tissue were analyzed as soon as possible after the death of the animal, in this case 70 minutes, and later at the end of $5\frac{1}{2}$ and $10\frac{1}{2}$ hours. In this experiment the glycogen was very rapidly transformed into dextrose, the change being practically complete at the end of $5\frac{1}{2}$ hours.

The changes in the total carbohydrate content of the samples of muscle during incubation are of great interest. All of the samples, which were incubated for periods ranging from $5\frac{1}{2}$ hours to 34 days and 3 hours, show large relative decreases in total carbohydrates, varying from 55.4 per cent in the sample incubated 2 days and 1 hour to 91.5 per cent in the sample incubated 27 days

and 1 hour. The sample incubated only $5\frac{1}{2}$ hours shows a decrease of 81.1 per cent.

By referring to Table II the column headed "glycogen and dextrose calculated as dextrose," it will be noted that there is first a large decrease in the total carbohydrate content of the sample incubated $5\frac{1}{2}$ hours; while in the sample incubated $10\frac{1}{2}$ hours there is a considerable increase as compared with the $5\frac{1}{2}$ hour sample, but still a marked decrease as compared with the first sample analyzed. This increase in the carbohydrate content of the sample incubated $10\frac{1}{2}$ hours, as compared with the sample incubated $5\frac{1}{2}$ hours, confirms a similar increase observed in Experiment 1. Finally the samples of biceps femoris muscle incubated for 27 days and 1 hour, and 34 days and 3 hours contain less carbohydrates than the sample incubated $5\frac{1}{2}$ hours.

On the whole, the results of this experiment show that the samples of muscular tissue examined had very marked glycolytic properties. It is of particular significance that glycolysis took place so rapidly immediately after the death of the animal. The fact that there was a decrease of 81.1 per cent in the carbohydrate content of the sample of muscular tissue analyzed $5\frac{1}{2}$ hours after the animal was killed, as compared with the amount present in the muscle 1 hour and 10 minutes after slaughter, indicates the rapidity with which the change took place.

Experiment 3.—The importance of analyzing the samples of tissue at frequent intervals following the death of the animal is at once apparent from an examination of the data presented in Table III. The changes in the glycogen content of the samples of the biceps femoris muscle during incubation are of particular interest. The sample analyzed 1 hour after the death of the animal contains 0.672 per cent glycogen, while the one analyzed 1 hour and 45 minutes later, or 2 hours and 45 minutes after the animal was killed, contains 0.930 per cent, or a relative increase of 38.4 per cent over the amount present in the first sample. The samples incubated for 4 hours and 15 minutes, and 6 hours and 20 minutes show gradually decreasing percentages of glycogen as compared with the sample incubated 2 hours and 45 minutes, but, on the other hand, larger percentages of glycogen than the sample analyzed 1 hour after slaughter. The samples incubated 10 hours and 30 minutes, and 27 hours contain less glycogen than

any of the others. These facts are of much importance in that they indicate that muscular tissue has the ability to synthesize glycogen. Since there was no decrease in the dextrose content of the muscle during the increase in glycogen, and because proof is lacking that glycogen can be formed from fat, it appears that the glycogen must have been formed from proteins.

The total carbohydrate content of the biceps femoris muscle shows, first, a relative increase of 33 per cent in the sample incubated 2 hours and 45 minutes as compared with the sample incubated 1 hour, and then increases of 21.4 and 12.0 per cent in the samples incubated 4 hours and 15 minutes and 6 hours and 20 minutes, respectively. The samples incubated for periods ranging from 8 hours and 30 minutes to 27 hours show decreases varying from 11.1 to 35 per cent. If the percentage changes in the total carbohydrate content of the samples of muscle be referred to the amount present in the sample incubated 2 hours and 45 minutes as a basis, it will be found that the samples incubated for periods ranging from 4 hours and 15 minutes to 27 hours show the following decreases in carbohydrates: 8.7, 15.7, 33.5, 51.0, and 33 per cent.

Unfortunately samples of the semimembranosus muscle were not analyzed at as frequent intervals as were the samples of the biceps femoris muscle. The samples of the semimembranosus muscle incubated for periods of 20 days and 4 hours and 28 days and 3 hours show decreases in total carbohydrates amounting to practically 60 per cent of the amount present in the sample analyzed 1 hour after slaughter. Judging by the results obtained in the other experiments, it is probable that the decreases in the carbohydrate content of the muscle just noted took place within a few hours after the death of the animal.

Taken as a whole, the results of this experiment indicate (1) samples of the biceps femoris muscle apparently had the ability to synthesize glycogen and at the same time to increase their content of total carbohydrates; (2) samples of the biceps femoris muscle showed marked glycolytic properties, over 50 per cent of the carbohydrates disappearing within a period of 8 hours; (3) samples of the semimembranosus muscle also had marked glycolytic properties.

Experiment 4.—Samples from both the biceps femoris and

semimembranosus muscles showed appreciable glycolytic properties. The decreases in the total carbohydrate content of the muscles did not proceed at a constant rate, the changes taking place most rapidly during the first 12 hours after the death of the animal. The total carbohydrate content of the biceps femoris muscle had decreased 18.7 per cent 3 hours after the death of the animal, 46.4 per cent after 12 hours and 15 minutes, and 59.8 per cent after 7 days and 5 hours. The semimembranosus muscle decreased 43.1 per cent in total carbohydrates in a period of 6 hours and 30 minutes following slaughter.

Taken as a whole, the results of this experiment show that the samples of muscular tissue examined had appreciable glycolytic properties, the disappearance of sugar taking place most rapidly within 12 hours after the death of the animal. These results confirm those obtained in the previous three experiments.

SUMMARY OF RESULTS.

The results of the experiments which have been reported show that muscular tissue from a normal ox has appreciable glycolytic properties, and that these properties are most active for a short time following the death of the animal. The factors which limit the glycolytic changes which take place in dead muscular tissue under such conditions as prevailed in these experiments have not been determined as yet, but in all probability the changes are limited by conditions associated with the development of rigor mortis.

Muscular tissue also appeared to have the ability to synthesize carbohydrates, both glycogen and dextrose, presumably from proteins.

These findings show that appreciable glycolysis may take place in muscular tissue without the aid of any extract from pancreatic tissue. These results do not, on the other hand, indicate that the pancreas does not play an important part in the glycolysis that takes place in living muscular tissue. In the experiments which have been reported the animals were bled as completely as possible, but the tissues were not washed free from blood, since this procedure did not seem practicable under the conditions of the experiments. It is barely possible, but highly improbable, that the small amount of blood which remained in the muscular tissue

may have supplied an activator or coenzyme previously secreted by the pancreas, which stimulated glycolysis. Rather, the authors are of the opinion that the pancreas functions somewhat differently in sugar metabolism. They are of the opinion that in all probability the pancreas plays an important part in the actual formation of the glycolytic enzymes in living tissues, but that active glycolysis may take place in dead tissues in the absence of any extract of the pancreas.

The possibility that the decrease in the copper-reducing action of the clarified extracts of the incubated samples of muscular tissue might be due to the formation of disaccharides, as suggested by Levene and Meyer (29), has been considered. In a considerable number of cases the copper-reducing action of clarified muscle extracts was determined both before and after inversion, but in no case was there any evidence of the formation of disaccharides.

Attention has been called to the somewhat irregular character of the changes in the carbohydrate content of the muscular tissue during autolysis. In a small degree such irregularities may be due to unavoidable errors, but for the most part they must be due to other causes. Considering the complex nature of the metabolism of sugar in the living organism, it is reasonable to expect that the changes which take place in the carbohydrates of dead muscle may be equally or even more complex. In the normal living organism the various forces work in harmony so as to dispose of the absorbed sugars according to the needs of the body; in the dead tissues the equilibrium of forces has been broken up and other factors enter into play, many of which are not clearly understood, so that the changes which take place under such conditions cannot compare either in orderliness or in extent with those that take place in the living organism.

Glycolysis has been measured in the experiments which have been reported by the disappearance of carbohydrates. The questions naturally arise: What has become of the carbohydrates? Have they been oxidized completely to carbon dioxide and water, as in the living organism, or has the oxidation been incomplete? The work of Stoklasa suggests that the sugar present in the muscular tissue may have been oxidized only to carbon dioxide and alcohol. In order to secure information on this subject the following experiments were conducted.

II.

The purpose of the experiments which are to be reported was to determine whether the glycolysis that takes place in muscular tissue results in the production of carbon dioxide and alcohol.

The general plan of these experiments was to secure sterile samples of muscular tissue by aseptic methods as soon as possible after the death of the animal, and to incubate the tissue in sterile media in suitable fermentation tubes so as to collect any gas that might be formed. Special large size fermentation tubes, of the so called "fish hook" type, having a capacity of 360 cc., were used. Samples of muscular tissue were taken in the special room previously described. Every precaution was taken to avoid contamination of the samples of tissue. A strict bacteriological control was exercised over all the experiments.

Experiment 5.—Fermentation tubes were filled with neutral plain beef broth to which had been added 1 per cent by volume of a solution containing 10 per cent of mixed phosphates (1 part monosodium phosphate, 2 parts disodium phosphate) to preserve neutrality, and which had been saturated with carbon dioxide. The filled fermentation tubes were held 3 days in an incubator at 37°C., and those which showed bacterial contamination were discarded. In this and subsequent experiments the fermentation tubes were removed from the incubator only a few minutes before the samples of tissue were to be placed in the tubes so that the temperature of the media would be close to 37°C.

A rabbit weighing 2,540 gm. was killed by severing the anterior aorta, and when bleeding was complete the carcass was immersed in a solution of mercuric chloride (1:1,000) until the hair was thoroughly wet. The skin was removed with as great care as possible to avoid contamination of the exposed tissues and the carcass was wrapped in a cloth previously soaked in a solution of mercuric chloride. In taking samples of muscular tissue for incubation, the proposed lines of incision were seared by means of a hot spatula, the surface tissue was trimmed back, and a sample of the underlying muscular tissue was taken out by means of a knife and pair of forceps, and transferred to a fermentation tube. A total of 5 samples weighing from 9 to 26 gm. were taken in this way. The first sample was taken 7 minutes after the death of the animal, and the last sample after 33 minutes. The tubes were then placed in an incubator at 37°C.

Immediately after the samples of tissue had been placed in the fermentation tubes it was noticed that a few small gas bubbles had collected on the surfaces of the pieces of tissue. After 1 hour from the time the rabbit was killed a few small bubbles had collected in the tops of the

closed arms of four tubes. After 2, 3, 5, 6, and 24 hours there had been no further production of gas. After incubation for 72 hours, two tubes were found to be contaminated. Since previous experiments have shown that glycolysis takes place most rapidly during the first 12 hours after the death of the animal, the fact that the incubated samples of muscular tissue gave rise to practically no production of gas during the first 24 hours indicates that carbon dioxide was not an end-product of any glycolysis that took place in the tissue. In view of the negative results, the fact that two tubes proved to be contaminated is immaterial.

Experiment 6.—A rabbit weighing 2,590 gm. was killed and skinned as in Experiment 5. The surfaces where the samples of muscular tissue were to be taken were not seared but simply wiped with cotton saturated with a solution of mercuric chloride (1: 1,000). The surface tissue was first trimmed back and a sample of muscular tissue was then taken out by means of a knife and pair of forceps, immersed in a sterile normal salt solution so as to remove mercuric chloride, and transferred to a fermentation tube previously filled with Locke's solution. This solution was made up according to the following formula:

	per cent
Sodium chloride	0.900
Calcium "	0.024
Potassium "	0.042
Dextrose	0.100

The first sample of muscular tissue was taken 15 minutes after the death of the animal, and the last sample 8 minutes later, when the tubes were at once transferred to an incubator at 37°C. Six samples were taken, the weights ranging from 36 to 75 gm.

A few minutes after the pieces of tissue had been placed in the fermentation tubes a few small gas bubbles were observed clinging to the surfaces of the pieces of tissue. After incubation for 1 hour and 4 hours there had been no further production of gas. After 5 hours the gas bubbles had practically all disappeared. At the end of 24 hours 4 tubes proved to be contaminated, and three of these showed abundant gas formation. The two sterile tubes showed no gas production.

Experiment 7.—This experiment was conducted in a similar manner to Experiment 6 and gave like results.

Experiment 8.—In this experiment samples of muscular tissue of an ox were taken by aseptic methods and transferred to fermentation tubes containing Locke's solution. The methods employed in the slaughter of this animal and the taking of the samples of tissue have been described under Experiment 4. The first sample of tissue was transferred to a fermentation tube 2 hours and 42 minutes after the death of the animal, and the last sample 17 minutes later when the tubes were placed in an incubator at 37°C. A total of six samples was taken. The tubes were examined after incubation for 50 minutes, 2½, 5, and 21 hours, and 2 days and 3 hours, but in no case was there any evidence of gas production. After incuba-

tion for 2 days and 3 hours the tubes showed no positive evidences of contamination, but cultures taken from the tubes showed that all samples were contaminated. Since there had been no gas production, the fact that all the tubes were contaminated is immaterial.

DISCUSSION OF RESULTS.

The results of the experiments which have been reported indicate that carbon dioxide is not an end-product of the glycolysis that takes place in muscular tissue autolyzed under aseptic conditions. The few small gas bubbles observed clinging to pieces of muscular tissue in several instances cannot be regarded as being of any significance. Since no carbon dioxide was formed it was unnecessary to test for alcohol. It is evident that the end-products of the glycolysis that took place under the conditions of these experiments are intermediate between dextrose, and carbon dioxide and water. The nature of these products remains to be determined, but our knowledge of the changes that take place in sugars in the living organism indicates very strongly the probable character of these intermediate products. The conditions necessary for the complete oxidation of sugars by dead muscular tissue are not clear.

CONCLUSIONS.

The results of the experiments which have been reported in this paper appear to justify the following conclusions.

1. Muscular tissue autolyzed under aseptic conditions shows appreciable glycolytic properties.
2. Glycolysis takes place most rapidly within a comparatively few hours after the death of the animal.
3. The glycolysis that took place under the conditions of these experiments did not result in the production of carbon dioxide.
4. Muscular tissue apparently has the ability to synthesize carbohydrates.
5. In part at least, and probably in their entirety, the processes by which sugars are utilized by the animal body are enzymatic in nature.

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TABLES FOR FINDING THE ALKALINE RESERVE OF BLOOD SERUM, IN HEALTH AND IN ACIDOSIS, FROM THE TOTAL CO_2 OR THE ALVEOLAR CO_2 OR THE pH AT KNOWN CO_2 TENSION.

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(Received for publication, May 30, 1917.)

The object of the present paper is the comparison of the results obtained by means of the hydrogen electrode and with alveolar CO_2 apparatus with those obtained by means of the Van Slyke apparatus for total CO_2 in plasma. Van Slyke has given us a factor for roughly finding the alveolar CO_2 from the plasma CO_2, but this does not help us interpret the determinations with the hydrogen electrode, and we hope that the following tables may elucidate the data on acidosis and give some basis for analysis of such investigations as those of Peters.

Since the alkaline reserve has been expressed in various units in papers by the senior author it is necessary to state that in the present paper we mean the sum of the equivalents of strong bases minus the sum of the equivalents of strong acid in the serum, expressed as a fraction of a normal solution. It is the excess base or titratable alkalinity, where the precautions described below are taken to insure the correct end-point in the titration, *viz.*, the pH of distilled water at the same CO_2 tension.

It appears to be approximately true that blood serum behaves as a bicarbonate solution made isotonic by the addition of NaCl in regard to pH and CO_2 tension (McClendon, *a*, *b*). That is to say, the non-volatile buffers, phosphates and proteins, do not have an easily measurable effect on the pH at 42 mm. CO_2 tension. The concentration of diffusible phosphates is about 0.001 M in serum, and failure to detect their effect on the pH is due to

their low concentration. The concentration of protein is less in serum than in plasma, but the sodium oxalate necessary to prevent coagulation of plasma is reduced to alkali by ashing in the compensation dialysis method of determining the alkaline reserve (*i.e.*, dialyzing serum against $NaHCO_3$ solutions made isotonic with NaCl and finding the solution not changed by dialysis). Hence we confined our studies to serum, in order to have a uniform material for all experiments.

EXPERIMENTAL.

A modified Ringer's fluid of the following composition behaves as serum of the same alkaline reserve in regard to pH, CO_2 tension, and total CO_2: 0.7 per cent NaCl, 0.04 per cent KCl, 0.02 per cent $CaCl_2$, 0.027 per cent $MgSO_4.7H_2O$, 0.2 per cent $NaHCO_3$, 0.001 M H_3PO_4. We have used this fluid in experiments on living animals with gratifying results. It should not remain open to the air, as it thus becomes too alkaline.

Our first problem was the determination of the alkaline reserve of the serum to be used in further work. We tried the compensation dialysis method of Michaelis and Rona, and found the alkaline reserve of ox serum apparently to vary from 0.03 to 0.04 N, but the osmotic pressure of the proteins and the varying permeability of the collodion membranes introduced errors, and our results may be too high. If serum is titrated with acid, using the hydrogen electrode as indicator, some means must be taken to remove the CO_2 or keep its tension constant in order to determine the correct end-point in the titration. If hydrogen is bubbled through the serum in an ordinary hydrogen electrode, some serum is carried away in the foam. In order to obviate this difficulty, we used the rotating hydrogen electrode previously used with bicarbonate solutions (McClendon, *b*). Volumetric flasks of the same size were filled with the serum and a different quantity of acid was added to each flask. The flasks were shaken and portions of the serum transferred successively from each flask to the rotating electrode, and a stream of the gas was passed through. The pH of the serum from each flask as well as the acid added was recorded, and these data were used to construct a titration curve from which the end-point in the titration could be

determined with accuracy (being the pH of distilled water at the same CO_2 tension). Owing to the time required for a single titration, we made no attempt to get the normal alkaline reserve of any species of animal by this means. The blood had been defibrinated in open dishes, and CO_2 allowed to escape. It is well known that the alkaline reserve of serum decreases when CO_2 escapes from the defibrinated blood, due to an ionic exchange with the corpuscles. The results reported apply to serum prepared as described.

Having determined the alkaline reserve of a series of sera, we added $NaHCO_3$ or acid to portions of serum to increase the range of alkaline reserve.

The experiments were made in a room kept at 20° by means of an electric apparatus. On a few days when the outside temperature rose above 20° the room was cooled by means of ice placed in front of an electric fan. A highly sensitive galvanometer was used with the potentiometer.

About 1.5 cc. of serum was placed in the rotating electrode, and a mixture of H_2 and CO_2 passed through it from the gas mixer. The stop-cocks were closed and the pH was determined, then the serum was used for the determination of the total CO_2 in the Van Slyke apparatus. In order to avoid some loss of CO_2 that always occurred when the serum was measured in a pipette, the serum was allowed to run quickly into the cup of the Van Slyke apparatus and a measured quantity allowed to run down into the graduated portion. The remainder of the serum was washed out of the cup with distilled water and the cup dried with filter paper. A correction was applied for the reversal in curvature of the meniscus and the volume of the hole in the stop-cock. 0.6 N HCl was introduced into the apparatus until the total fluid was 2.5 cc. This concentration of HCl has about the same absorption coefficient as serum for CO_2 (0.822 at 20°). The determination was that recommended by Van Slyke. The CO_2 pumped out was absorbed by NaOH and the CO_2 remaining in the acidified serum was calculated in each determination, then the total CO_2 was reduced to 0° and 760 mm.

The results of the experiments are given in Figs. 1 and 2. From Fig. 1 the pH may be determined from the CO_2 tension and alkaline reserve, or the CO_2 tension may be determined from the

pH and alkaline reserve, or the alkaline reserve may be determined from the pH and CO_2 tension.

The alkaline reserve may be approximately determined from the alveolar CO_2 tension alone, since the pH of blood in the arteries is remarkably constant, being about 7.33–7.5 (the variation being due largely to the method of determination). If the serum is allowed to come to equilibrium with the alveolar air at 20° the

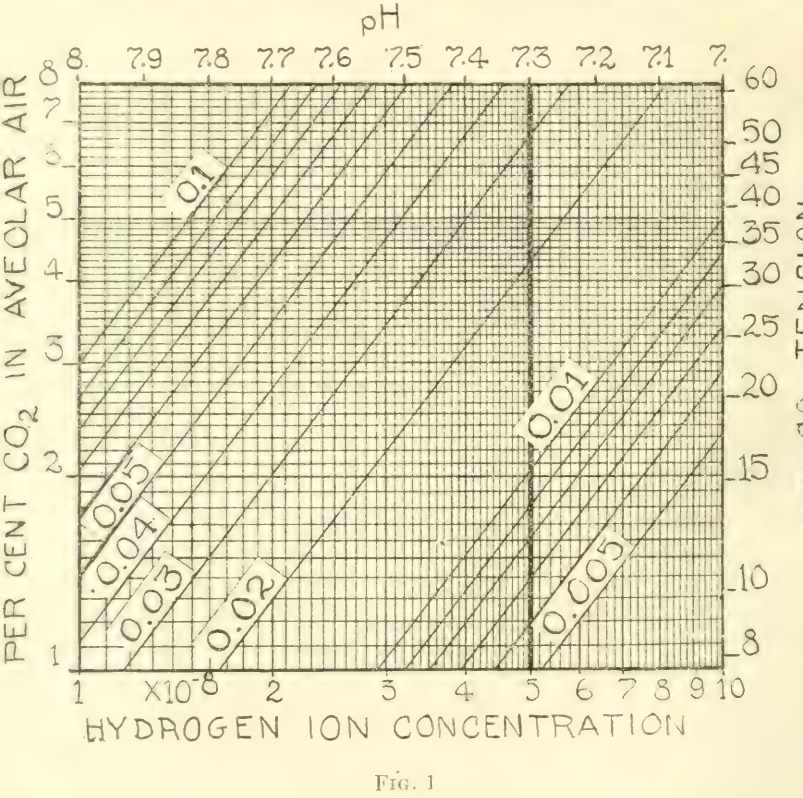

Fig. 1

pH is about 0.15 lower and in our experiments was 7.3; hence the alkaline reserve is represented by that diagonal passing through the intersection of the alveolar CO_2 abscissa with the pH 7.3 ordinate. In other words the alkaline reserve is nearly half the alveolar CO_2 tension in atmospheres, and can be determined more accurately than this from Fig. 1. In order to bring the blood or serum at 20° to the same pH as in the arteries, its CO_2 tension

must be lowered about 25 per cent. If the blood is drawn from the artery directly into a tube that may be closed so as to prevent

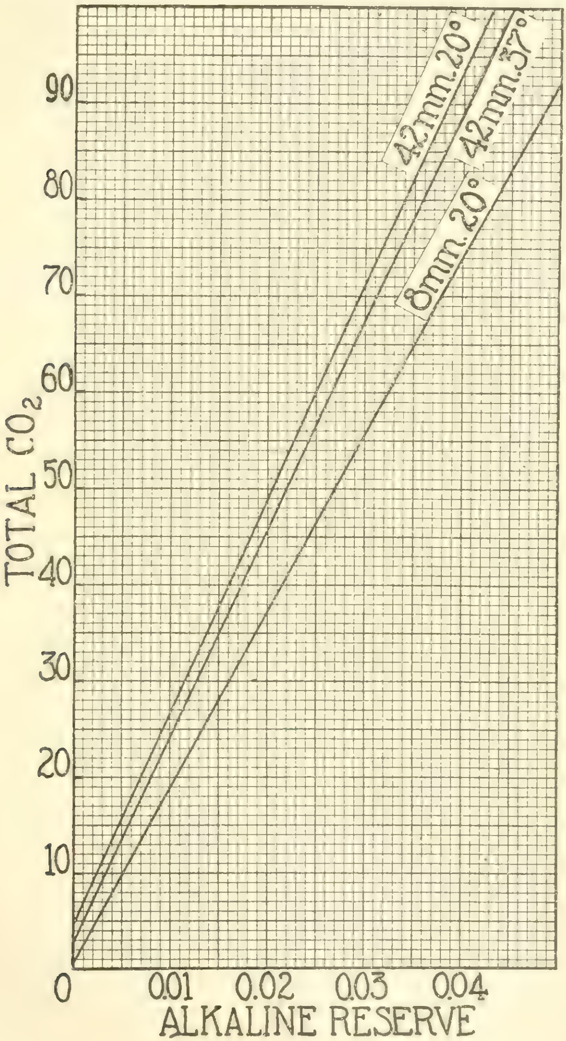

Fig. 2

gain or loss of CO_2 and cooled to 20°, the pH remains the same but the CO_2 tension falls about 25 per cent. Fig. 1 would represent the relations at 37° if the numbering of the pH scale at the

top were increased by about 0.15, or so that the heavy ordinate is numbered about pH 7.45 instead of 7.3. That is to say, the numerical relations between alkaline reserve and CO_2 tension remain the same, and it seems unnecessary to draw another figure for 37°. The rise of 0.15 pH due to rise in temperature from 20° to 30° at constant CO_2 tension is uncertain. We obtained values from 0.11 to 0.17 on serum, blood, and bicarbonate solutions. For a given difference of temperature the rise is less the higher the initial temperature.

Because war conditions have separated the authors from each other and from some of their notes, it is impossible to give the results in detail, but it seems worth while to report at present the mean of the results obtained.

From Fig. 2 the alkaline reserve may be determined from the total CO_2 at a given CO_2 tension and temperature, but a slight error in tension or temperature has little effect on the total CO_2. (The effect of change in temperature was calculated from a large number of experiments on bicarbonate solutions.) The data may be condensed into the following empirical formulas:

20°, 42 mm. CO_2 tension, total CO_2 = 4.5 + 2,180 × alkaline reserve.
20°, 8 " " " " " = 0.87 + 1,820 × " "
37°, 42 " " " " " = 2.9 + 2,155 × " "

If arterial blood is drawn into a tube in such a way that the serum may be collected without loss of CO_2, the alkaline reserve may be calculated from the total CO_2, by using the formula:

pH 7.5, total CO_2 = 2,250 × alkaline reserve.

If serum is subjected to alveolar CO_2 tension at 20° the alkaline reserve may be calculated from the formula:

pH 7.3, total CO_2 = 2,333 × alkaline reserve.

If the CO_2 is removed from serum until the pH rises to 8, the alkaline reserve may be calculated from the formula:

pH 8, total CO_2 = 1,850 × alkaline reserve.

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Since this paper was written the attention of the senior author has been called to a paper by Hasselbalch (*Biochem. Z.*, 1916, lxxviii, 112). Hasselbalch's observation that rise of temperature does not change pH if CO_2 does not escape was previously observed by McClendon and Magoon (and is also true of bicarbonate solutions). Hasselbalch's observation that rise in temperature at constant CO_2 tension increases the pH of bicarbonate solutions is also true of blood. His observation that corpuscles behave as buffers was previously published by McClendon and Magoon and is explained perhaps by the ionic exchange between corpuscles and plasma. Obviously the pH of the corpuscle interior is not measured by the hydrogen electrode, but a change in pH of corpuscle may cause a change in pH of plasma or serum.

THE EFFECT OF TEMPERATURE ON THE REACTION OF LYSINE WITH NITROUS ACID.*

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(Received for publication, July 12, 1917.)

Lysine, unlike the rest of the amino-acids with exposed amino groups, requires a considerably longer period to react completely with nitrous acid. This has been demonstrated by Van Slyke in two papers.[1] The reason for this is explained by the possible slower reactivity of the ε-amino group. In the first paper it was reported that the reaction is not complete until 30 minutes, but the second paper would indicate that the reaction goes to completion in 15 minutes. In the Van Slyke method of protein analysis it is, therefore, recommended to shake the hexone bases for half an hour, so as to be absolutely sure of the completion of the reaction of lysine.

It was incidentally noted by Dr. Roxas, working in this laboratory last summer, that at high temperatures lysine reacts with both amino groups in 5 minutes. This observation invited further research upon the influence of temperature on the reaction of lysine with nitrous acid.

In order to demonstrate clearly the meaning of our data, Van Slyke's results on the behavior of lysine towards nitrous acid are quoted below.

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[1] Van Slyke, D. D., *J. Biol. Chem.*, 1911, ix, 199; 1912, xii, 282.

TABLE I.

*Reaction of Lysine with Nitrous Acid (Van Slyke).**
(For Each Analysis 0.0888 Gm. of Lysine Picrate Was Used.)

Time of reaction.	N gas.	Temperature.	Pressure.	Amino N.	Calculated.
min.	*cc.*	*°C.*	*mm.*	*per cent*	*per cent*
5	9.90	19	758	6.36	7.47
15	10.75	19	758	6.87	7.47
15	11.20	20	758	7.06	7.47
30	11.50	19	758	7.39	7.47
30	11.80	20	758	7.54	7.47
50	11.70	19	758	7.51	7.47

* Van Slyke, *J. Biol. Chem.*, 1911, ix, 199.

TABLE II.

*Reaction of Lysine with Nitrous Acid (Van Slyke).**

Time.	Weight.	N gas.	Temperature.	Pressure.	Amino N.	Calculated.
min.	*gm.*	*cc.*	*°C.*	*mm.*	*per cent*	*per cent*
5	0.2	25.4	24	764	7.13	7.47
15	0.2	26.7	24	764	7.49	7.47
30	0.2	26.7	24	764	7.49	7.47

* Van Slyke, *J. Biol. Chem.*, 1912, xii, 282.

The interesting fact in connection with Van Slyke's work indicated above is that at 19°C., 6.36 per cent amino or 84.01 per cent of the total amino nitrogen reacted in 5 minutes, while at the higher tenperature, 24°C., 7.13 per cent amino or 95.2 per cent of the total amino nitrogen reacted in the same period of time.

EXPERIMENTAL.

Lysine picrate was prepared according to the Kossel and Kutscher method, which on analysis proved to be pure. Roxas' observation was corroborated by working with lysine picrate in an incubator room at 37°C. It will be noted from the following table that *both amino groups reacted in 5 minutes.* In view of this consideration, it appeared important to determine the lowest temperature at which *both* amino groups would react in 5 minutes,

the idea being to reduce the time of shaking the hexone bases when working at high temperatures.

Due to the practical inconvenience of handling the Van Slyke apparatus in a thermostat, the use of the latter as a means of controlling temperature was not attempted. The temperatures lower than 37°C. were obtained by the use of an electrical hot plate placed a short distance from the apparatus. This work was conducted in a small room and the temperatures desired were easily controlled within 1°. The data are recorded in Table III.

TABLE III.

Effect of High Temperatures on the Reaction of Lysine with Nitrous Acid.

Weight.	Time.	Temperature.	Pressure.	N gas.	Weight.	Amino N.	Amino N, per cent of total.
gm.	*min.*	*°C.*	*mm.*	*cc.*	*mg.*	*per cent*	
0.04000	5	37	741	5.95	3.0035	7.51	99.60
0.04000	5	37	741	5.95	3.0035	7.51	99.60
0.03636	5	29.5	746	4.92	2.6019	7.16	94.96
0.03636	5	32	746	5.25	2.7483	7.55	100.10
0.03636	10	30	746	5.28	2.7438	7.54	100.00

It will be noted that 32°C. is the lowest temperature for the completion of the reaction in 5 minutes; also that 30°C. is the lowest temperature for the completion of the reaction in 10 minutes.

Attention was next directed to the effect of low temperatures on the reaction of lysine with nitrous acid. To secure these results advantage was taken of the winter months, and after several trials, constant low temperatures within 1–2° were obtained by open windows. Fully 10 minutes at the constant low temperature were allowed to elapse before shaking was started. It was found impossible to work below 1°C. on account of the appearance of ice in the burette of the apparatus. The data are given in Table IV.

It will be observed that low temperatures have a peculiar retarding effect on the reaction of lysine with nitrous acid; also that at 0–1°C. only 50 per cent of the total amino nitrogen reacted. It seems that at this low temperature and at the low concentration employed (0.36 to 0.40 per cent) only one-half, or probably

TABLE IV.

Effect of Low Temperatures on the Reaction of Lysine with Nitrous Acid in 5 Minutes.

Weight.	Temperature.	Pressure.	N gas.	Weight.	Amino N.	Amino N, per cent of total.
gm.	*°C.*	*mm.*	*cc.*	*mg.*	*per cent*	
0.03636	5–6	748.5	3.15	1.8881	5.19	68.83
0.03636	4–5	748.5	3.00	1.8057	4.97	65.94
0.03636	3–4	748.5	2.85	1.7225	4.74	62.86
0.03636	0–1	748.5	2.25	1.3767	3.78	50.10
0.03636	0–1	748.5	2.25	1.3767	3.78	50.10
0.03636	0– −1	748.5	2.15	1.3209	3.63	48.14

the α-amino group reacted, and that the ϵ-amino group was inactive.

Since, according to Van Slyke and Birchard,[2] it is the exposed ϵ-amino group of lysine in the protein molecule which makes the protein directly reactive to nitrous acid, it was of interest to investigate the effect of low temperatures on the reaction of several proteins with nitrous acid. Casein,[3] dissolved in dilute sodium carbonate, and gliadin[4] (in more concentrated solution), dissolved in dilute sodium hydroxide, were employed, as outlined by Van Slyke and Birchard. Although an appreciable volume of gas was obtained at room temperatures in 5 minutes, almost a negligible volume of gas (within the limits of experimental error) was obtained in the cold. Zein, which is reported as containing no lysine, gave a very small volume of gas at room temperature.

This phase of the work was conducted in a refrigerator room and very satisfactory conditions for controlling temperature were afforded. It will be noted that the ϵ-amino group, the only reacting group in this case, is considerably retarded, almost excluded, at the low temperatures.

In the process of the retardation of the reaction of lysine with nitrous acid at the low temperatures employed, there is a possi-

[2] Van Slyke, D. D., and Birchard, F. J., *J. Biol. Chem.*, 1913–14, xvi, 539–547.

[3] 1 gm. of casein was dissolved in 100 cc., and 2 cc. portions were used in the micro apparatus.

[4] 5 gm. of gliadin were dissolved in 100 cc., and 10 cc. portions were used in the large apparatus.

TABLE V.

Effect of Temperature on the Reaction of Proteins with Nitrous Acid in 5 Minutes.

Protein.	Weight.	Temperature.	Pressure.	N gas.	Amino N.
	gm.	*°C.*	*mm.*	*cc.*	*mg.*
Zein..................	0.02	25	740.5	0.06	0.00272
"	0.02	25	740.5	0.06	0.00272
Casein..............	0.02	27	740.5	0.63	0.33667
"	0.02	27	740.5	0.64	0.34201
"	0.02	3	748.0	0.05	0.03020
"	0.02	5	748.0	0.21	0.12580
Gliadin.............	1.00	24	746.0	1.10	0.60225
"	1.00	25	746.0	1.20	0.65700
"	1.00	0–1	748.0	0.05	0.03069
"	1.00	0–1	748.0	0.05	0.03069

bility of the additional retardation due to the α-amino group of the lysine molecule. In order to determine whether low temperatures have a retarding effect on the α-group of amino-acids, the effect of low temperatures on the reaction of alanine with nitrous acid was tried. The results are given in Table VI.

TABLE VI.

Effect of Temperature on the Reaction of Alanine with Nitrous Acid.

Weight.	Temperature.	Pressure.	N gas.	Weight.	Amino N.	Calculated.
gm.	*°C.*	*mm.*	*cc.*	*mg.*	*per cent*	
0.012	23.5	743	3.48	1.90216	15.90	15.73
0.012	23.5	743	3.48	1.90216	15.90	15.73
0.012	3.0	745	3.28	1.97170	16.47	15.73
0.012	3.0	743	3.28	1.91250	15.91	15.73

It will be noted that low temperatures have no retarding effect on the reactivity of the α-amino group of alanine. It is, therefore, safe to conclude from the preceding results that *low temperatures have a retarding effect on the ϵ-amino and not on the α-amino group of lysine.*

SUMMARY.

1. At definite concentrations, 32°C. is the lowest temperature at which both amino groups of lysine react with nitrous acid in 5 minutes.

2. At certain definite concentrations, 30°C. is the lowest temperature at which both amino groups of lysine react with nitrous acid in 10 minutes.

3. It is suggested that at temperatures of 30°C. and above, 10 or a maximum of 15 minutes would be more than sufficient for shaking the hexone bases in the Van Slyke method of protein analysis, at any concentration, instead of 30 as was the practice heretofore.

4. At temperatures of 1°C. and slightly below and at definite concentrations it is possible to render the ϵ-amino group of lysine entirely inactive.

METHODS FOR THE DETERMINATION OF BLOOD SUGAR IN REFERENCE TO ITS CONDITION IN THE BLOOD.

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(Received for publication, July 11, 1917.)

In a previous article von Hess and McGuigan (1) studied the condition of the sugar in the blood and concluded that it is all free and exists in the blood as in a water solution. They realized that anesthesia may change the blood sugar in amount and probably in condition, and to control this as far as possible they used various anesthetics, but always obtained the same results. While everything points to the sugar being free there still remains the possibility of a loose combination of sugar, similar to that of oxygen with hemoglobin, which might be disrupted by such mild manipulations as they used in their dialysis work. The present work was undertaken to determine whether or not we could find any basis for such an assumption. At some stages of the work it seemed we had evidence of such a combination, but closer investigation gives a more satisfactory explanation and confirms the previous conclusion, that the blood sugar is free in solution. There are some differences, however, between the reactions of dextrose in a water solution and dextrose in a protein-free blood filtrate. It is this difference we think which is mainly responsible for the great variations reported in the literature regarding the normal level of the sugar in the blood.

In the present work we have used both the Bertrand (2) and the Lewis-Benedict (3) methods. Under some conditions there is a great discrepancy in the results of the two methods and an examination of this difference we think throws light on the cause of the reported variations in the normal level of the blood sugar. While engaged in this work we noticed the article by W. B. Smith

(4) showing that picric acid does not interfere with the reaction of Fehling solution. If such be the case it renders the Bertrand and Benedict methods directly comparable since the conditions of precipitation can be made exactly alike. The differences, however, between the analyses of the blood picric acid filtrate by the two methods were so enormous that we doubted the truth of the statement. However, we found in control work, with dextrose in water, that picric acid does not interfere with the Bertrand method, as the following results show: A solution of dextrose was prepared, approximately 0.1 per cent *in water* and determined by the Bertrand method with the results: normal, 0.110, 0.110 per cent. After the addition of 1 gm. of picric, an amount greatly in excess of saturation but which went into solution on boiling, the results were: 0.113, 0.115 per cent. This slight increase may be considered negligible and within the limits of error. It is constant, however, and due to a salt action, as salts to some extent increase the reduction of alkaline copper, as the results of Bertrand show.

The influence of salt on the result of the Bertrand method is shown by the following figures:

Sugar in water.	Sugar in salt.			
I.	II. 2 per cent Na_2SO_4	III. 5 per cent Na_2SO_4	IV. 12 per cent Na_2SO_4	V. 25 per cent Na_2SO_4
per cent	*per cent*	*per cent*	*per cent*	*per cent*
0.084	0.085	0.090	0.110	0.110
0.085	0.090	0.094	0.110	
0.085			0.110	
0.085			0.110	
0.085			0.110	

Beyond 12 per cent the influence of the salt does not change. We quote this salt, and 10 to 20 per cent, because hitherto in the precipitation of the proteins from blood, we have used sodium sulfate and acetic acid to a great extent. The influence of the acetic acid will be emphasized later. The most probable explanation of the salt action is its influence on the boiling point of the solution.

Having convinced ourselves that picric acid does not interfere with the results of the Bertrand method, and what slight influence it has is to increase the yield of sugar, we were prepared to investigate the differences in the results of the Bertrand and the Lewis-Benedict methods. The comparison here is very direct since we use picric acid to remove the proteins in each case.

Methods.

To obtain blood, dogs were bled from the jugular vein with a hypodermic syringe. The blood was usually oxalated, sometimes defibrinated, at other times used directly without oxalate after quickly measuring before clotting could occur. 10 cc. samples were taken for each separate determination. This amount was for convenience only, as 5 cc. are sufficient. The blood was laked by adding it directly to 60 cc. of water. After 15 minutes laking 1 gm. of solid picric acid was added and thoroughly mixed with the blood solution until saturation. The volume was then made to 150 cc. with saturated picric acid in water, filtered, and 100 cc. of the filtrate (6.66 cc. of blood) were used for the analyses. This makes a convenient volume to handle, it filters rapidly, and is sufficiently large to minimize slight changes due to evaporation on boiling. The picric solution was brought to boiling, and to it were added 60 cc. of mixed Fehling solution (also boiling), and the boiling was continued for 2 minutes, timed with a sand glass, then filtered through asbestos, washed free from picric acid with hot water, and the whole transferred to a beaker with about 40 to 50 cc. of hot water. To this were added 25 cc. of 5 per cent ferric sulfate in 20 per cent sulfuric acid, and this solution was titrated with permanganate, while still hot. Each specimen should be titrated at about the same temperature and under the same conditions.

The potassium permanganate solution used was standardized by preparing a water solution of dextrose of 0.1 per cent, and 10 cc. of this were placed in 90 cc. of water, or if using salt to precipitate proteins, in the same strength of salt as used in the experiment, and the permanganate was brought to such a strength that 1 cc. represents 0.001 gm. of glucose.

The Lewis-Benedict method was carried out as recommended by Meyers and Bailey (5). The following table shows some of the results we have obtained by these methods directly, and also by Bertrand's method after "hydrolysis."

Any method of removal of the proteins of the blood, where acid is avoided, will give results similar to these quoted. We had corroborated the work by the use of (1) alcohol, (2) methyl alcohol, (3) colloidal iron, (4) sodium sulfate, (5) basic lead acetate, (6) dialysis of the serum.

Bertrand, direct.	Lewis-Benedict.	Bertrand, after "hydrolysis," or directly with 5 per cent KOH, Fehling solution.
0.010	0.100	0.067
0.010	0.080	0.090
0.030	0.119	0.110
0.054	0.114	0.116
0.058	0.102	0.096
0.030	0.095	0.092
0.035	0.100	0.100
0.023		0.085
0.015	0.080	0.075
0.030		0.123
0.020	0.094	0.100
0.020	0.075	0.090
0.010	0.078	0.075
0.022	0.114	0.117
0.017	0.090	0.196
0.015		0.120
0.018	0.080	0.112
0.018		0.130
Average 0.0241	0.0944	0.0991
Comparable average......0.015	0.0944	0.0954

In seeking for the cause of the great difference between the Bertrand and the Benedict methods we naturally suspected the presence of a sugar combination—"virtual sugar"—or a sugar of the maltose type, and so hydrolyzed and obtained a rise in the amount, and a figure which in most cases agrees very closely with the Benedict method. There were many facts, however, which made us doubt that there was an actual hydrolysis. First, to get the highest results, we had to use a certain amount of acid which reduced the alkalinity from 12.5 per cent KOH to 5 per cent, and second, the time necessary for the completion of the supposed hydrolysis was very short, and higher results were obtained if the acid used was not neutralized before applying the Fehling solution. Finally we found if the acid were added directly to the Fehling solution, *i.e.*, lessened alkalinity of the Fehling, the same results were obtained as after the supposed hydrolysis. It is evident, therefore, that lessened acidity and not hydrolysis is

the true explanation. The reason for not neutralizing after hydrolysis and before using the Fehling was due to the fact that there is great variability in the alkaline strength of Fehling's solution as recommended in standard works, and even without neutralizing we had still a strongly alkaline liquid that corresponded to many so called Fehling solutions. Controls with this solution tested in water solution gave good results. The results in Table III were obtained by adding 4 cc. of acetic acid to the 100 cc. of picric acid filtrate of the blood solution used for the analysis.

The alkalinity of Fehling's solution has been given (6) as 125 gm. of KOH in 500 cc. Mathews' (7) and many other texts adopt this amount. The United States Pharmacopeia VII used the same amount but in Edition VIII reduced this to 75 gm., and in the most recent edition, IX, reduces this to 50 gm. in 500 cc. This makes the alkalinity of the mixed fluids 5 per cent KOH in the last case against 12.5 per cent in the earlier editions. Claude Bernard at first used about 11.5 per cent NaOH and later about 6 per cent of the mixed fluids. Fehling's original solution (8) was about 5.6 per cent NaOH, which he afterward reduced to about 4.75 per cent NaOH. At the present time we may say the alkalinity of the complete Fehling's solutions as recommended varies from about 5 per cent KOH or less to 12.5 per cent NaOH. It is because of this great variation that we were in the beginning somewhat careless in neutralizing the acid added for hydrolysis and we were justified in this because a water solution dextrose yield is but slightly changed even by great variations in the alkalinity of the Fehling. *In a protein-free blood filtrate, however, the condition is vastly different.* Benedict's and Bertrand's methods with dextrose in water solution agree exactly, but note the difference when they are applied for the determination of the sugar in the blood.

In the preceding cases and throughout, except as mentioned, we used a Fehling solution of the following composition:

I.		II.	
$CuSO_4$	34.65	Rochelle salt	173
Water to	1,000 cc.	NaOH	125
		Water to	1,000 cc.

Except for the substitution of NaOH for KOH, this solution has the composition of that recommended by Bulletin 107 since they advise dilution with an equal quantity of water before use. It is also of the same composition so far as the alkali goes as recommended by Hammarsten (9).

With a water solution of dextrose almost theoretical results can be obtained with this solution and Bertrand's method as we have used it. The above results are so striking and so out of harmony with current opinion that errors in method, or the presence of interfering bodies, must be seriously considered. Traces of protein or other bodies which hold cuprous oxide in solution were thought of. The presence of such bodies seems improbable, as is shown by the following work.

First, dextrose added to normal or to diabetic blood can be recovered. This has been shown in many cases. Second, the result of dialysis shows that whatever the nature of such a body may be, it is dialyzable. Dialysis does not preclude peptone bodies which perhaps would be the protein that would interfere most. Such a body, however, would seem to be excluded by the lessening of the alkalinity which removes the effect and which could not remove the interfering body. While the presence of peptone thus seems improbable, its possibility still remains, for we know that traces of peptone beyond detection or recognition markedly influence the Bertrand method, while they have little influence on Benedict's method. If such a body be present, however, its solvent effect is limited because added sugar can be recovered and changes in the blood sugar are easily recognized. Third, the action of ether. In anesthetized animals, and in diabetics we can detect no change in the blood protein. Here again, however, the presence of a disturbing body might well escape us because the amount of sugar in these cases is so large that a change which would make a large percentage change in normal blood would be relatively insignificant here.

Instead of a protein the interfering body might be a dextrin-like body, but the fact that it will dialyze and is so easily "hydrolyzed" removes the probability of a dextrin.

Quite recently Scott (10) has suggested the presence of a lecithin combination, but this seems improbable since extraction of the picric filtrate or of the blood with ether before precipitation does not change the sugar in any way. This, however, does disprove not
Scott's contention, as he was working under different conditions. It is also not a glycogen-like body because when alcohol is used to precipitate the proteins, the glycogen would be removed, and yet we obtain results *similar* to the picric acid method.

The only investigator, so far as we know, who has reported the normal level of the blood sugar nearly as low as the figures we give for the direct determination after precipitation by picric acid is Shaffer (11). In the early part of the work we were much influenced by Shaffer's results and statements, and thought that our picric acid method showed that most of the sugar was in a combined form. Further work, however, has convinced us that this is erroneous, that the previous work of von Hess and McGuigan is correct, and that Shaffer also was unaware of the influence of highly concentrated alkaline Fehling's solutions on protein-free blood filtrates. Shaffer used the 12.5 per cent KOH Fehling.

Effect of Ether Anesthesia on Blood Sugar.

Dog No.	Condition.	With and without ether anesthesia.	Sugar, using KOH. 12.5 per cent.	5.0 per cent.
			per cent	*per cent*
1	Eck fistula 2 months old	None.	0.027	0.100
	" " 2 " "	3 hrs.	0.132	0.237
2	Reversed Eck fistula 6 weeks old	None.	0.020	0.090
	" " " 6 " "	3 hrs.	0.090	0.180
3	Eck fistula, healed	None.	0.040	0.160
	" " "	3 hrs.	0.180	0.210
4	Normal	None.	0.056	0.078
	"	10 min.	0.115	0.133
5	"	None.	0.066	0.102
	"	10 min.	0.073	0.140
6	"	None.	0.020	0.110
	"	30 min.	0.040	0.140
	"	120 "	0.080	0.156
7	"	None.	0.052	0.104
	"	3 hrs.	0.205	0.260
8	"	3 "	0.125	0.163
9	"	3 "	0.090	0.125
10	"	3 "	0.142	0.210
11	"	3 "	0.075	0.108

His average for the normal blood sugar in four dogs was 0.036, 0.020, 0.046, and 0.026 per cent, figures that agree closely with ours by the direct picric acid method. His results after anesthesia show a considerable rise, showing also, we think, agreement with our explanation that added sugar by whatever cause can be recovered. The low results he at first obtained we think are due to the solvent action of the strong alkali on the cuprous oxide which is increased by an unknown body in the blood. This solvent action is fully satisfied by the amount of sugar in the normal blood so that added sugar or the increase due to anesthesia may be recovered. This solvent action is much greater in a blood filtrate than in a water solution.

The influence of ether is thus seen to increase the amount of sugar when determined by the Bertrand method after precipitation of the proteins by picric acid. This action is not due to the breaking up of a sugar combination because blood extracted directly with the ether is not changed, nor is the picric blood filtrate changed by similar treatment. The experiment also shows that Eck fistula does not seem to change the action of ether on the blood sugar.

Morphine also increases the sugar as determined by this method, as is shown by the following experiment.

	12.5 per cent KOH.	5 per cent KOH.
Normal dog. Blood sugar direct.	0.015 per cent	0.08 per cent
3 hrs. after 0.15 gm. of morphine.	0.165 " "	0.225 " "

The opinion has been expressed by more than one writer that the sugar in the blood exists in combination with something of the nature of an amboceptor. There are some facts brought out by the picric acid precipitation of proteins that might be used to sustain this theory. Since picric acid shows that the blood sugar is not a simple solution of glucose in water, its use also permits the testing of the amboceptor hypothesis and to a considerable extent supports that theory.

For example, Shaffer found that dextrose added to a solution of sugar that had fermented with yeast could not be completely recovered. We have been able to confirm this as the following experiment will show.

2 gm. of cane sugar were dissolved in 500 cc. of tap water, one-half of a cake of yeast was added, and the whole was placed in an incubator at 38°C. for 48 hours. After this time:

1. Direct test with Fehling solution showed no dextrose present.
2. 100 cc. boiled with 4 cc. of acetic acid and Fehling solution showed without neutralization a trace reduction = 0.012 per cent.
3. 10 cc. of dextrose = 0.12 per cent dextrose added and 78 per cent of it recovered.
4. The same boiled with 4 cc. of acetic acid = 100 per cent recovered.

Sugar added to a yeast fermented residue, however, can be recovered or lost, depending on the alkalinity of the Fehling solution used.

One-half of a cake of yeast was added to 500 cc. of 0.2 per cent dextrose solution and the mixture allowed to stand for 1 week in an incubator at 40°C. A known amount—0.1 per cent—of dextrose was then added. When determined with Fehling's solution of the strength given by the Pharmacopeia, which is 5 per cent KOH, this showed in water solution 0.105 per cent; with 12.5 per cent NaOH, Fehling, 0.034 per cent. Increasing the copper in Fehling solution has the same influence as decreasing the alkali. In the preceding case when 12 per cent NaOH was used and 7 per cent $CuSO_4$ instead of 3.5 per cent, we obtained 0.09 per cent; 20 per cent NaOH and 7 per cent $CuSO_4$, 0.1 per cent.

This variation in the copper content of Fehling's solution is better shown in the following experiment.

A dog was bled under ether and the blood defibrinated and precipitated with picric in the usual way. The alkaline tartrate part of the Fehling is constant, 125 gm. of KOH and 346 Rochelle Salt in one liter, but the copper solution varies as indicated.

2	per cent	Cu,	0.021	per cent	dextrose	in	the	blood.
4	"	"	0.044	"	"	"	"	"
8	"	"	0.096	"	"	"	"	"
12	"	"	0.157	"	"	"	"	"

At this last strength of copper the black oxide is precipitated so that the last figure is not reliable.

With the same strength of Fehling's solution as tested against a known dextrose solution in water the variation is much less, *e.g.*, a 0.102 per cent dextrose solution gives:

2	per cent Cu,		0.07	per cent.	
4	"	"	" 0.188	"	"
8	"	"	" 0.102	"	"
12	"	"	" 0.102	"	"

Since the utilization of the dextrose by the yeast cell is probably by the same mechanism as in the animal cell, this masking or combining with the sugar suggests a similar preliminary step in the animal body. Accordingly we have tested blood by:

1. Adding sugar directly to it when fresh, and determining the amount of the added dextrose that could be recovered.
2. The same addition after blood had undergone glycolysis.
3. Normal and diabetic blood has been compared.

In the normal blood the *added* sugar can be recovered, while in blood that has been freed from sugar by glycolysis added sugar is immediately masked as in yeast fermentation and cannot be recovered until after the addition of acid. This suggests that the blood sugar normally is in combination, and that when the sugar is removed by glycolysis, the amboceptor (12) still retains the power to unite with free dextrose converting it into the same form as the dextrose normally existing in the blood.

In like manner we have determined the sugar in normal blood directly by the picric acid Bertrand method, and have found that all the added sugar can be recovered, while in blood that has undergone glycolysis a certain amount combined with the amboceptor and can be recovered only after adding acid or reducing the alkalinity. The following experiments illustrate this.

Experiment No.	Normal sugar. By using Fehling 12.5 per cent NaOH.	After adding acid.	Sugar added in:	Recovered.
	per cent			
I	0.01		0.102 per cent water.	0.124
	0.01		0.102 " " "	0.125
II	0.02		0.102 " " picric.	0.142
III	0.023	0.085	0.098	0.100
IV	0.017	0.123	0.102	0.158

The recovery of more sugar than is added is, we think, due in part to the liberation of acid radicles when Cu_2O is formed. Thus H_2SO_4 then acts as so much added acid and sensitizes the Fehling solution, which then reacts with some of the potential sugar; and

in part it is due to the fact that weaker alkali in the Fehling normally gives a higher result.

After Glycolysis.

Experiment No.	Time of glycolysis.	Sugar added.	Recovered.
	hrs.		
I	48	0.102	0.046
	48	0.102	0.060
II	18	0.102	0.075

In normal blood by the picric acid method the average blood sugar direct is about 0.01 per cent. In sensitizing the Fehling by the addition of acid this amount rises to about 0.10 per cent. If dextrose be added to the normal filtrate containing 0.01 per cent we recover more than is added and this is probably because the reduction of the Cu causes a liberation of acid which sensitizes Fehling's solution by reduction of the alkalinity.

Normal blood was glycolyzed until it showed no sugar when tested with the Fehling solution. The Benedict method showed 0.016; Fehling containing 2.5 per cent NaOH or 10 per cent Na_2CO_3 gave 0.01 per cent. If now to 100 cc. of this glycolyzed blood picric acid filtrate we add 10 cc. of dextrose solution—or 0.102 per cent—and determine how much of this can be recovered by varying the alkalinity of Fehling's solution, we find for the original undiluted Fehling—12.5 per cent NaOH—only 60 per cent recovered, or 0.060. 5 per cent NaOH gives 0.110; 2.5 per cent gives 0.120 per cent. We thus find that added sugar can be recovered if not too much alkali is added or if sodium carbonate be used instead of KOH.

Controls with water solution gave with 12.5 per cent NaOH 0.102 per cent; with 5 per cent NaOH, 0.110 per cent. With the weaker alkali consequently we recover the total sugar, added and actual.

The following results show how changing the alkalinity influences the yield of the sugar as determined by Fehling's solution—Bertrand.

NaOH	Dog's blood in Fehling.
per cent	*per cent*
12.5	0.018
5	0.090
4	0.105
2.5	0.122

The Lewis-Benedict method on this blood gave 0.106 per cent.

Increasing the amount of copper in the Fehling has the same effect as decreasing the alkalinity, as the following analysis of dog's blood will show.

$CuSO_4$ per cent	12.5 per cent NaOH. per cent
2	0.021
4	0.044
8	0.096
12	0.157

In the last case some black oxide is precipitated.

A solution of dextrose in water or in picric acid solution is not nearly so much influenced by this variation of the copper, or by changes in the alkalinity.

$CuSO_4$ per cent	12.5 per cent NaOH. per cent
2	0.070
4	0.088
8	0.102
12	0.102

The Difference between Normal and Diabetic Blood.

We have examined the blood of several normal and diabetic persons which, at one stage of the work, we thought showed a significant difference. When examined more closely we think no such difference exists. The ratio of the amount of sugar after hydrolysis, or that determined by the 5 per cent KOH Fehling is striking.

Ratio in Normal Dogs.

Direct.	After hydrolysis or by 5 per cent KOH Fehling.	Ratio.
0.048	0.140	1:3
0.010	0.067	1:7
0.028	0.070	1:2.5
0.025	0.092	1:4
0.020	0.110	1:5
Average of 12		1:4

The influence of anesthesia, as is well known, raises the blood sugar, and if continued long enough may cause a glycosuria. It also changes this ratio remarkably.

I. Normal	0.020	0.110	1:5
After 2 hours ether anesthesia	0.08	0.156	1:2
II. 10 min. ether	0.052	0.104	1:2
III. After 3 hrs. ether	0.205	0.260	1:1.2

Ether always reduces this ratio, likewise anything that raises the amount of sugar in the blood. Averaging six dogs under ether for 1 hour, the ratio fell from 1:4 to 1:1.3. In a case of adrenalin glycosuria without ether the ratio was practically 1:1. In one case of phlorhizin diabetes, in which, as is well known, the blood sugar does not increase, the ratio did not change.

In normal human beings the ratio is practically the same as in normal dogs, as the following table will show.

No.	Indirect.	Hydrolyzed.	Ratio.
I	0.025	0.090	1:3.6
II	0.035	0.160	1:4.8
III	0.082	0.230	1:3
IV	0.082	0.240	1:3

Human diabetic blood again shows almost a ratio of 1:1 as the following will show.

No.	Indirect.	Hydrolyzed.	Ratio.
I	0.172	0.256	1:1.5
II	0.290	0.307	1:1.05
III	0.670	0.730	1:1.1

Some of this diabetic blood was allowed to glycolyze in the incubator at 40°C. until the sugar had all disappeared, and then a known amount of glucose was added. Of the added glucose in two cases only 40 and 60 per cent was recovered when analyzed by the strong alkali Fehling. So that if there be such a thing as an amboceptor, it acts, so far as we can make out, as strongly in diabetic blood as in the normal.

If we analyze the cases above cited it will be seen that, while the ratio differs enormously, the actual difference between the sugar obtained by the direct picric acid precipitation and after hydroly-

sis is about the same in all cases. For instance in the worst diabetic the difference between 0.730 and 0.670 is 0.06, and this is approximately the figure in most of the cases where the ratio is 1:4 or 1:5. Whatever the cause may be it acts in all cases but is more striking in those cases with an initial low figure. Since normally strong alkali to some extent dissolves cuprous oxide, in blood filtrates this solvent action is much greater than in water solution. The cause is not known. It is, however, organic, not inorganic, because blood ash added to sugar has no such influence.

SUMMARY AND CONCLUSIONS.

An investigation of the blood sugar was undertaken with special reference to its condition in the blood and to the difference between normal and diabetic blood. It was found that picric acid does not interfere with the use of Fehling solution or its modifications. This allows a direct comparison of the two most used methods, the Lewis-Benedict and the Bertrand. By the use of picric acid to remove proteins and the subsequent use of the Bertrand method it was found that when a Fehling solution which contains 12.5 per cent KOH is used very low sugar yields were obtained. In some cases only about one-tenth of that was obtained by the Benedict method. When 5 per cent KOH Fehling was used results agreeing closely with the Benedict method were obtained. The difference is not due to the picric acid directly because picric acid in water solution has no influence and other methods of removing the protein give similar results. Alcohol, colloidal iron, and sodium sulfate all give considerably lower results with the higher alkaline Fehling. The picric acid, however, is the most pronounced. Solutions of dextrose in water are somewhat lower with the stronger alkali Fehling but this difference is relatively small in comparison with the differences in the blood filtrate. The cause of the difference has not been definitely decided but apparently it is something of an organic nature which is not removed by the picric acid. Perhaps it is in the nature of, or at least acts in a manner similar to, creatine or peptone. It is not because the stronger alkali destroys sugar under these conditions because after boiling with the stronger alkali Fehling the higher yield of sugar can be obtained by the addition of acid suf-

ficient to reduce the alkali from 12.5 per cent to 5 per cent KOH. Also with added sugar, or by increase in the sugar content of the blood as in diabetes, or in anesthesia, or in adrenalin glycosuria and similar conditions, the whole increase can be obtained, which would not be the case if the alkali were destroying the sugar. This recovery of the increased sugar greatly changes the ratio obtained by the use of the two concentrations of alkali or between the results of the stronger alkali and the result of the Benedict method. This ratio roughly changes from 1:4 to 1:1. If this ratio has any significance it relates to the amount of interfering substance in the blood under the different conditions. Whatever this body is, it exerts very little influence on the Benedict method and this method seems to be the one of choice for blood sugar determination. The Bertrand method carried out as we have used it also gives good results but the strength of the alkali in the Fehling solution is important and should not be over 5 per cent KOH. We think the varying strength of the alkali used is the most important cause for the great variation in the normal level of the blood sugar reported in the literature. This level for man and dogs as originally reported by Bernard is about 0.1 per cent. The present work corroborates the work of von Hess and McGuigan, showing that the sugar in the blood reacts as it does in a water solution.

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THE ORIGIN OF CREATINE. II.*

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If arginine is partly converted into creatine by the animal tissues, glycocyamine may be regarded an intermediate product in this reaction.

$$\begin{array}{l} NH_2 \\ | \\ C = NH \\ | \\ HN.CH_2.CH_2.CH_2.CHNH_2.COOH. \end{array} \longrightarrow$$

$$\begin{array}{l} NH_2 \\ | \\ C = NH \\ | \\ HN.CH_2.CH_2.CH_2.COOH. \end{array} \longrightarrow \begin{array}{l} NH_2 \\ | \\ C = NH \\ | \\ HN.CH_2.COOH \end{array}$$

Therefore it is important to determine whether the animal organism possesses the power to convert glycocyamine into creatine. Considerable work has been done on this problem but the question has not been conclusively decided. Czernicki,[1] using the zinc chloride precipitation method, obtained an increase of creatinine in the urine of rabbits after feeding glycocyamine but not when glycocyamidine was fed. This author does not regard his work as decisive. Jaffé[2] also determined the sum of creatine and creatinine in the urine and muscle of rabbits after feeding and injecting glycocyamine. He also employed the Neubauer zinc chloride method. This author found that glycocyamine, when

* The experimental data are taken from a dissertation submitted by Harry M. Hines as a partial requirement for the degree of Master of Science, State University of Iowa, Iowa City.

[1] Czernicki, W., *Z. physiol. Chem.*, 1905, xliv, 294.

[2] Jaffé, M., *Z. physiol. Chem.*, 1906, xlviii, 430.

injected or fed, led to an increase in the creatine and creatinine fraction both in muscle and urine. Dorner,[3] a pupil of Jaffé, repeated the latter's experiments but employed the more accurate colorimetric method. He found an increased excretion of creatine after the administration of glycocyamine, but also found that muscle *in vitro* possessed the power to methylate glycocyamine. Mellanby[4] criticizes Jaffé's technique and Dorner's experiments. He finds no consistent increase in the creatine content of chicken muscle after the administration of glycocyamine, but regards his work as inconclusive. More recently Palladin and Wallenburger[5] have apparently obtained relatively large increases in creatine when rabbit's muscle was permitted to act on glycocyamine at incubator temperature, and also when rabbits were injected with glycocyamine.

Our work on this problem may be divided into three parts. At first it was necessary to see whether creatine could be accurately determined in the presence of glycocyamine according to the conventional methods. We found that an acid solution sufficiently strong to convert creatine into creatinine would usually effect a partial conversion of glycocyamine into glycocyamidine, and as the latter gives the picric acid reaction, the results for creatine were too high. Both Jaffé and Dorner separated creatine and creatinine from glycocyamine by exhaustive extraction with alcohol. We find that 95 per cent alcohol will extract sufficient quantities of glycocyamine to interfere with the creatine determinations. The solubility of glycocyamine in 95 per cent alcohol is 0.02 per cent, that is 20 mg. dissolve in 100 cc. of boiling 95 per cent alcohol.

The second part of our work was concerned with the action of freshly hashed muscle on glycocyamine *in vitro*. Under these conditions, an increase of creatine was never encountered.

Finally we resorted to injection, perfusion, and feeding experiments on rabbits, dogs, and men. While the results of these experiments were not uniform, we obtained, in some cases, an increased elimination of creatine after the injection of glycocyamine.

[3] Dorner, G., *Z. physiol. Chem.*, 1907, lii, 225.

[4] Mellanby, E., *J. Physiol.*, 1908, xxxvi, 447.

[5] Palladin, A., and Wallenburger, L., *Compt. rend. Soc. biol.*, 1915, lxxviii, 111. This reference was only obtainable in abstract form.

EXPERIMENTAL.

The glycocyamine was obtained through the interaction of guanidine and chloroacetic acid according to the procedure of Ramsay.[6] The yield was 65 per cent, based on the amount of chloroacetic acid used. The guanidine required for this reaction was synthesized from calcium cyanamide, according to the excellent method of Levene and Senior.[7]

Determination of Creatine in the Presence of Glycocyamine.

Palladin and Wallenburger determined the creatine in muscle by heating the extract on a water bath for 3 hours in an approximately 0.6 N hydrochloric solution as suggested by Riesser.[8]

Experiment A.—100 cc. of a 1 per cent solution of glycocyamine in approximately 0.6 N hydrochloric acid were heated on a boiling water bath for 3 hours, then diluted to 200 cc. To one of two flasks, each containing 5 mg. of creatinine, were added 5 cc. of the glycocyamine solution (25 mg. of glycocyamine), and the intensity of the color was compared, after the addition of picric acid and alkali. The color of the glycocyamine flask was deeper than the control. This difference was equivalent to 0.8 mg. of creatinine for 25 mg. of glycocyamine or 3.2 for 100 mg.

Experiment B.—To two of four flasks, each containing a known quantity of creatine dissolved in 275 cc. of approximately 0.6 N sulfuric acid were added 100 mg. of glycocyamine. After heating on the boiling steam bath for 3 hours, the intensity of the Jaffé reaction was determined by means of the colorimeter and expressed as creatinine.

With	glycocyamine	38.97 and	39.36 mg.,	mean 39.16
Without	"	35.8 "	35.9 "	" 35.85

Difference, for 100 mg. of glycocyamine, 3.31 mg.

Experiment C.—A similar experiment in which 0.66 N sulfuric acid was used, showed an increase corresponding to 3.9 mg. of creatinine per 100 mg. of glycocyamine.

Experiment D.—A fourth experiment in which diluted muscle extract and 0.66 N sulfuric acid was used showed that 100 mg. of glycocyamine increased the color proportionately to 4 mg. of creatinine.

Solubility of Glycocyamine in Boiling 95 per Cent Alcohol.—An excess of analytically pure glycocyamine was boiled with 95 per cent alcohol under reflux for 1 hour, then filtered through a hot steam jacketed funnel into a tared flask of known volume.

[6] Ramsay, H., *Ber. chem. Ges.*, 1908, xli, 4385.

[7] Levene, P. A., and Senior, J. K., *J. Biol. Chem.*, 1916, xxv, 623.

[8] Riesser, O., *Z. physiol. Chem.*, 1913, lxxxvi, 415.

After evaporation of the alcohol on the steam bath, the flask was dried to constant weight and weighed. It was found that 57 cc. of boiling 95 per cent of alcohol dissolved 0.0115 gm. of glycocyamine or 100 cc. 0.020 gm.

Weyl's Reaction.—Both Jaffé and Dorner placed considerable importance on Weyl's test as a means of determining glycocyamidine in the presence of creatinine. We were unable to obtain sharp reactions in the concentrations with which we were dealing, so we preferred to control our experiments as described below.

Experiments in Vitro.—Freshly hashed rabbit muscle or dog liver was weighed into a flask containing glycocyamine dissolved

Weight of muscle.	Time at 38°C.	Glycocyamine added.	Creatine.	Weight of muscle.	Time at 38°C.	Glycocyamine added.*	Creatine.
gm.	hrs.	mg.	per cent	gm.	hrs.	mg.	per cent
Experiment E.				Control.			
7.293	24	40	0.577	7.529	24	40	0.546
7.724	24	40	0.561	8.672	24	40	0.564
7.996	48	33	0.596	7.714	48	40	0.596
6.426	48	40	0.595	7.061	48	40	0.600
7.337	48	40	0.563				
Average			0.578	Average			0.576
Experiment F.				Control.			
7.146	24	50	0.461	7.081	24	50	0.470
6.784	46	50	0.470	6.093	46	50	0.471
Average			0.465	Average			0.470
Experiment G.				Control.			
Weight of liver.				Weight of liver.			
6.370	36	50	0.0567	6.129	36	50	0.0587
9.039	42	50	0.0444	8.384	42	50	0.0520
7.147	63	50	0.058	8.105	106	50	0.056
Average			0.053	Average			0.056

* Glycocyamine added at the time of the determination.

in isotonic Henderson's phosphate solution. After the addition of toluene, the flask was placed in the incubator for from 24 to 96 hours and the creatine content determined according to the method of Janney and Blatherwick.[9] The absence of bacterial growth was confirmed by culture. The controls which had remained in the incubator were analyzed after the addition of an equivalent amount of glycocyamine. In this way the color due to glycocyamine was accounted for. As is evident from the tables, our results do not indicate methylation of glycocyamine *in vitro* by muscle or liver.

Injection and Feeding Experiments.

Rabbits were placed in a metabolism cage on a diet of carrots. The urine was gathered in 24 hour periods and diluted to a defi-

Experiment H.—A gray rabbit weighing 2,540 gm. was used for this experiment. At the conclusion of the experiment, the muscle contained 0.452 per cent of creatine, 72.55 per cent of water, and 3.71 per cent of nitrogen.

Experiment H.

Day.	Creatinine.	Creatine.*	Glycocyamine injected.	Day.	Creatinine.	Creatine.*	Glycocyamine injected.
	mg.	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*
2	84	2	0	12	97	0	120
3	120	6	0	13	100	11	400
4	93	10	0	14	99	39	350
5	77	2	0	15	102	44	350
6	110	2	0	16	108	54	350
7	62	22	0	17	93	62	350
8	83	9	0	18	102	61	100
9	86	1	0	19	99	54	200
10	112	1	0	20	100	51	0
11	91	0	0	21	112	40	0
				22	106	15	0
Average..	91.8	5.5		Average.	101.6	39.2	
				23	107	2	0
				24	106	4	0

* Creatine is expressed as creatinine.

[9] Janney, N. W., and Blatherwick, N. R., *J. Biol. Chem.*, 1915, xxi, 567.

nite volume. During the second period, they were injected with known amounts of glycocyamine subcutaneously. Creatine was determined by heating the urine on the water bath with 0.66 N sulfuric acid for 3 hours.

A quantity of glycocyamine greater than that which could be present in the urine of the injected animals was added to normal rabbit urine and its effect on the creatine estimation determined. It was found that this quantity of glycocyamine (400 mg.) increased the daily creatine from 10 to 12 mg.

If we allow 12 mg. for the effect of dissolved glycocyamine in the urine and 6 mg. for the average daily excretion during the preliminary period, we still have an excess of more than 21 mg. to be ascribed to the influence of glycocyamine.

Experiment I.—A female pregnant rabbit[10] was placed in a cage on a diet of carrots. At the beginning of the experiment it weighed 2,435, at the end 2,564 gm. The creatine content of its muscle at the conclusion of the experiment was 0.448, the water content 74.22, and the nitrogen 3.58 per cent.

Experiment I.

Day.	Creatinine.	Creatine.*	Glycocyamine injected.	Day.	Creatinine.	Creatine.*	Glycocyamine injected.
	mg.	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*
1	60	6	0	10	112	4	300
2	106	5	0	11	92	5	150
3	88	11	0	12	76	37	300
4	84	3	0	13	116	39	300
5	84	7	0	14	71	35	300
6	124	12	0	15	111	30	300
7	71	1	0	16	74	17	0
8	111	2	0				
9	72	0	0				
Average...	89	5		Average..	93	24	
				17	103	0	0

* Creatine expressed as creatinine.

[10] The pregnant condition of this animal was determined when the experiment was in progress.

If we allow 12 mg. for the glycocyamine effect and 5 mg. for the daily average excretion during the preliminary period, 7 mg. remain which we are inclined to ascribe to the effect of the glycocyamine.

We are unable to account for the low concentration of creatine in the muscle tissue.

Experiment J.—The subject of this experiment was a male rabbit weighing 2,174 gm. at the beginning and 2,155 gm. at the end of the experiment. The diet consisted of carrots.

Experiment J.

Day.	Creatinine.	Creatine.*	Glycocyamine injected.	Day.	Creatinine.	Creatine.*	Glycocyamine injected.
	mg.	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*
1	74	4	0	9	105	10	150
2	78	0	0	10	68	7	300
3	57	4	0	11	96	4	150
4	50	6	0	12	85	25	300
5	53	1	0	13	Lost.		300
6	100	8	0	14	71	31	300
7	63	3	0	15	87	16	300
8	116	1	0	16	103	19	0
				17	53	25	0
Average...	74	3.4		Average..	83	17	
				18	90	0	0

* Creatine is expressed as creatinine.

After making the necessary deductions it is found that the excretion of creatine is increased during the second period by 1.6 mg. per day.

Experiment K.—The subject of this experiment was a bitch weighing 12 kilos. She was placed in a metabolism cage and fed on a mixture of skimmed milk powder, bread, lard, and agar. The experiment was started after the dog had been on this diet for 4 months. The urine was collected in 24 hour periods and made to a definite volume with the wash water from the cage. At the close of the experiment the dog weighed 15 kilos. 17 days later a small piece of the vastus externus muscle was removed from a right hind leg under anesthesia. It contained 0.35 per cent creatine,

73.71 per cent water, and 3.68 per cent nitrogen. 2 months later the corresponding muscle from the opposite side contained 0.318 per cent of creatine, 73.39 per cent of water, and 3.52 per cent of nitrogen.[11]

Experiment K.

Day.	Creatinine.	Creatine.*	Glycocyamine injected.	Day.	Creatinine.	Creatine.*	Glycocyamine injected.
	mg.	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*
1	386	1	0	10	362	14	400
2	370	15	0	11	345	64	600
3	382	21	0	12	396	61	830
4	380	23	0	13	398	60	800
5	378	12	0	14	380	90	800
6	345	3	0	15	365	122	800
7	393	29	0				
8	364	14	0				
9	364	14					
Average	373	14.5		Average	374	68.5	

* Creatine is expressed as creatinine.

In previous experiments we found that 100 mg. of glycocyamine when heated with 0.66 N acid for 3 hours reduces alkaline picric acid proportionately to 3 mg. of creatinine. Assuming that all of the glycocyamine which was injected appeared in the urine we would deduct 24 mg. for the glycocyamine effect and 14.5 mg. for the daily average creatine elimination during the preliminary period. This leaves a daily average elimination of 30 mg. to be ascribed to the effect of glycocyamine injection.

Experiment L.—A man weighing 62 kilos, aged 23 years, and in perfect health, was placed on a practically creatine-free diet consisting of bread, milk, cereals, vegetables, and fruit. The urine was carefully collected in 24 hour periods. Creatine was determined by heating the urine on the water bath for 3 hours with N sulfuric acid. Glycocyamine was administered *per os*.

[11] In previous unpublished experiments we have found that the creatine content of dog muscle is markedly diminished after the animals have been kept in cages on the milk and bread diet for several months.

Experiment L.

Day.	Creatinine.	Creatine.*	Glycocyamine fed.	Day.	Creatinine.	Creatine.*	Glycocyamine fed.
	mg.	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*
1	1,397	26	None.	10	1,358	81	1,000
2	1,376	15	"	11	1,362	24	2,000
3	1,378	23	"	12	1,342	33	2,000
4	1,398	27	"	13	1,282	83	2,000
5	1,320	15	"	14	1,342	53	3,000
6	1,214	0	"				
7	1,386	13	"				
8	1,367	57	"				
9	1,256	0	"				
Average	1,343	19.5		Average	1,337	55	

* Creatine is expressed as creatinine.

As considerable glycocyamine was undoubtedly present in the urine, the increased excretion of creatine during the second period is more apparent than real and is probably due to the presence of the guanidine acetic acid.

Experiment M.—A brown bull bitch weighing 12.3 kilos was fed on a diet consisting of skimmed milk powder, dry bread, lard, and agar, for 4 months prior to the operation. On March 7 a piece of muscle was removed from the right hind leg under aseptic precautions, and ether anesthesia. On March 14 the animal was given 200 mg. of glycocyamine twice a day and this was repeated for the next 8 days. On March 28 a corresponding piece of muscle was removed from the opposite side. The specimens of muscle were examined for creatine, water, and nitrogen.

Experiment M.

	Creatine.	Creatine per gm. of dry muscle	Water.	Nitrogen.
	per cent	*mg.*	*per cent*	*per cent*
Before glycocyamine................	0.30	9.2	67.31	3.72
After "	0.31	11.0	71.84	3.44

We are unable to account for the difference in water content in the two samples of muscle, as the weight of the dog remained almost constant throughout the experiment. The increase of creatine is not convincing as we have no means of saying how much glycocyamine has been stored in the muscle tissue.

Experiment N.—A small dog, 9 months of age, weighing 4.5 kilos, was decerebrated under anesthesia and a piece of the vastus externus muscle was removed from one hind leg. Approximately 2 gm. of glycocyamine dissolved in isotonic acid phosphate solution were injected into the jugular vein. $5\frac{1}{2}$ hours later a similar piece of muscle was removed from the opposite side. The creatine and water content of the muscle before the injection were 0.305 and 72.46 per cent, respectively, and after the injection, 0.316 and 71.47 per cent, respectively. In terms of dry substance, the creatine content of both samples is identical.

Experiment O.—The right hind leg of a dog weighing 15 kilos was perfused for 1 hour with 1.7 gm. of glycocyamine dissolved in 270 cc. of perfusion fluid. This consisted of 1.7 gm. of glycocyamine dissolved in 100 cc. of water, containing 3.28 cc. of 5 N hydrochloric acid. This was rapidly neutralized with an equivalent amount of sodium hydroxide solution and added to 150 cc. of the dog's own defibrinated blood. The pressure of the perfusate during the experiment fluctuated between 80 and 100 mm. of mercury. Only 110 cc. of fluid were recovered. The hamstring muscles were removed from both hind legs at the close of the experiment, and creatine and water determined. Creatine was determined according to the method of Janney and Blatherwick.[9] The perfusion technique has already been described.[12] Owing to its slight solubility in water, glycocyamine is difficult to perfuse in concentrations greater than that employed in this experiment.

Experiment N.

	Creatine.	Creatine per gm. of dry muscle.	Water.
	per cent	*mg.*	*per cent*
Perfused muscle	0.382	14.8	74.2
Unperfused "	0.374	13.4	72.12

The muscle of the perfused limb weighed exactly 1 kilo. If we assume that all of the glycocyamine contained in the perfusate was retained by the muscle tissue then 25.8 gm. of dry muscle tissue should contain 170 mg. of glycocyamine in addition to the creatine. If we deduct the chromogenic equivalent of this amount of glycocyamine,[13] which is 11 mg., from the apparent

[12] Baumann, L., and Marker, J., *J. Biol. Chem.*, 1915, xxii, 49.

[13] Janney and Blatherwick use 1 N acid for the conversion of creatine into creatinine. When 100 mg. of glycocyamine are heated with this concentration of acid for 3 hours, and alkaline picric acid is added to the neutralized solution a color develops which is equivalent to 5.5 mg. of creatinine.

creatine value for the perfused muscle, we find that 25.8 gm. of dry muscle contain 371 mg. of creatine or 14.4 mg. per gm. This increase is worthy of note. We hope to have opportunity to repeat this experiment.

CONCLUSIONS.

1. Our experiments do not offer any evidence for the methylation of glycocyamine by muscle or liver tissue *in vitro*.
2. The injection of glycocyamine into rabbits and dogs may be followed by an increased excretion of creatine.

THE PRODUCTION OF CREATINURIA IN NORMAL ADULTS.

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As a result of numerous investigations made in the thirteen years which have elapsed since the publication of Folin's method for the determination of creatine and creatinine, it is now a generally accepted fact that creatine does not occur in the urine of normal men living on a creatine-free diet. Until a few years ago it was believed that this statement applied also to normal non-pregnant women.

In 1911 it was however pointed out by Krause[1] that the urine of normal women frequently contains small amounts of creatine. This creatinuria is, according to this investigator, associated with the sexual cycle, is always present after menstruation, and while in some individuals it may disappear during the intermenstrual period in others it persists.

In work recently published from this laboratory,[2] it has been shown that in children it is possible to increase or decrease the amount of creatine excreted in the urine by increasing or decreasing the quantity of protein (creatine-free) in the food. As a result of these experiments the suggestion was made that creatinuria in normal children is probably due to the high level of their protein intake. If this theory be true, it would seem possible that if the protein intake could be increased to a sufficiently high level, creatinuria in normal adults might also be obtained.

In order to test out this hypothesis we have carried our experiments on four normal adults, two men and two women, along the same general lines and by the same methods used in investigating this subject in connection with children.

[1] Krause, R. A., *Quart. J. Exp. Physiol.*, 1911, iv, 293.

[2] Denis, W., and Kramer, J. G., *J. Biol. Chem.*, 1917, xxx, 189.

Our first experiments were carried out with two normal women who had previously acted on several occasions as subjects for metabolism studies. Just previous to becoming subjects for this investigation these women had, in connection with another problem, been living for about 8 days on a creatine-free diet of low protein content. The urines passed on the last day of this period were examined for creatine but with negative results. Both subjects were then placed on a creatine-free diet containing the largest amount of protein they could take.

The diets used, which were the same for both subjects, were as follows:

High Protein Diets.

	1	2	3
Eggs	12	6	12
Gelatin, *gm*	50		50
Cheese (neufchatel), *gm.*	200	50	200
Milk, *cc*	500	500	200
Bread, *gm.*	300	400	
Sugar, "	50	50	
Butter, "	50	50	
Lemons			2
Orange			1
Tomato and lettuce salad			One portion (not weighed).

Low Protein Diet.

Bread, *gm* 50	Baked apple, *gm* 200	Cream, 40 per cent, *cc* 200
Corn meal, *gm* 75	Apple and celery salad, *gm* 200	1 orange.
Potato, *gm* 250	Butter, *gm* 50	1 banana.
Lactose, *gm* 100	Bacon fat, *gm* 100	1 lemon.

The low protein diet was eaten by Subject I in the form given above; in the case of Subject II the corn meal was replaced by 200 gm. of potato.

From the results presented in Table I it will be seen that it was possible in the case of these two women to induce creatinuria by high protein feeding. After 5 days on the high protein Diet 1 Subject I was allowed to return for 3 days to the food which she ordinarily consumes, which consists largely of bread, vegetables,

and fruit, with a small amount of meat (approximately 50 gm.) once a day. Even this diet was apparently sufficiently low in protein to cause an immediate disappearance of all creatine

TABLE I.

Date	I. Normal female, 38 years old, weight 91.0 kg.				II. Normal female, 22 years old, weight 53.6 kg.			
	Total nitrogen.	Preformed creatinine.	Creatine.	Diet.	Total nitrogen.	Preformed creatinine.	Creatine.	Diet.
May	gm.	gm.	gm.		gm.	gm.	gm.	
14–15					5.02	1.12	0	
15–16	5.8	1.11	0		8.46	1.12	0	1
16–17	12.6	—	—	1	16.87	1.13	0	1
17–18	19.76	1.31	0.12	1	24.58	1.25	0.13	1
18–19	16.27	1.20	0.09	1	20.06	1.22	0.37	1
19–20	20.35	1.33	0.16	1	24.43	1.21	0.35	1
20–21	20.00	1.46	0.21	1	19.60	1.19	0.22	1
21–22	12.28	1.21	0	Mixed.*	12.66	1.28	0.24	Mixed.*
22–23	7.98	1.13	0	Mixed.*	18.45	1.12	0.18	1
23–24	9.80	1.13	0	Mixed.*	21.88	1.16	0.13	1
24–25	11.79	1.21	0	2	20.59	1.18	0.15	1
25–26	15.41	1.23	0	2	21.28	1.21	0.18	1
26–27	12.25	1.18	0.10	2	22.65	1.18	0.17	1
27–28	11.94	1.10	0.12	2	—	—	—	1
28–29	14.97	1.19	0.13	2	17.22	1.05	0.14	Low.
29–30	8.25	1.14	0.10	Low.	7.59	1.10	0.10	"
30–31	6.66	1.21	0	"	6.58	1.10	0.10	"
31–June 1	6.25	1.17	0	"	7.75	1.08	0.09	"
June								
1–2	5.48	1.11	0	"	4.65	1.00	0	"
2–3	5.90	1.11	0	"	5.76	1.07	0	"
3–4	5.98	1.12	0	"	5.88	1.16	0	"
4–5	14.61	1.38	0.08	3	12.95	1.19	0.09	3
5–6	18.69	1.42	0.15	3	17.26	1.13	0.28	3
6–7	21.08	1.26	0.20	3	23.85	1 09	0.34	3
7–8	20.50	1.28	0.22	3	22.22	1.33	0.20	3

* Ordinary mixed diet with meat, once a day.

from the urine. An attempt was then made by feeding the high protein Diet 2 to obtain an idea as to the general level at which creatinuria could be induced but even on this moderate protein intake creatine again appeared.

A low protein diet was then consumed for 5 days with the resultant disappearance of all creatine after the 2nd day, and this was in turn followed by a 5 day period on a diet containing approximately the same amount of protein as that contained in the high protein Diet 1, but differing from Diet 1 in that it contained only an extremely small amount (probably not more than 2 or 3 gm.) of carbohydrate.

By this experiment it was believed that it might be possible to obtain evidence as to the effect, if any, produced by the ingestion of carbohydrate on creatine excretion. As will be seen creatine appeared on the 2nd day as was the case in the period in which the high protein Diet 1 (which contained 300 gm. of bread) was taken and remained at about the same level as that reached during the first period.[3]

An attempt was made to follow this period of high protein and low carbohydrate feeding by one containing the same amount of protein but with a much larger amount of carbohydrate than was given in the first period. This, however, proved to be impossible, as the large amount of carbohydrate consumed (300 gm. of lactose and 300 gm. of bread) produced in both subjects such marked digestive disturbances that they were compelled to abandon the experiment.

The experimental periods on Subject II were essentially similar to those just described for Subject I except that the high protein Diet 1 was continued for a much longer period than in the case of Subject I, and that no period of moderate protein feeding was introduced.

Having been successful in the production of creatinuria in women by forced protein feeding we next attempted to duplicate our results in men.

Our third subject was a man 25 years old, weighing 62.6 kg. For a period of 14 days he consumed the following ration daily.

Whole eggs	6	Bread, *gm*	200
Egg whites	4	Macaroni, "	50
Gelatin, *gm*	50	Potato, "	100
Cheese (American), *gm*	100	Sugar, "	40
Milk, *cc.*	100	Butter, "	40

[3] During this period the urine was examined daily for acetone bodies by means of the Scott-Wilson reagent. In the case of Subject I a minute

During this period the urine was frequently examined for creatine but invariably with negative results. The mixed urines for the period were found to contain 251.1 gm. of nitrogen, or an average excretion of 18.0 gm. per day. An attempt to increase the protein intake was unsuccessful.

For our next experiment we used Subject IV, a man 20 years old, weighing 57.7 kg., who seemed possessed of a more capacious appetite than Subject III. For 12 days this man was kept on the same rations as had been used for Subject III. The mixed urines for the period were found to contain 227.9 gm. of nitrogen, or an average daily excretion of 19.1 gm.

Frequent attempts to find creatine in these urines were, however, invariably rewarded with negative results.

It was therefore decided to increase the protein intake, and for 5 days the ration consisted of:

Eggs	30	Chocolate, *gm.*	50
Milk, *cc.*	1,500	Bread "	150
Gelatin, *gm.*	50	Sugar "	50

The determination of the urinary nitrogen gave the following results for these 5 days: 22.9, 19.4, 32.7, 34.5, and 33.5 gm. In spite of the high level of nitrogen consumption no creatine could be detected in the urine at any time during the 5 days during which the experiment was continued.

In the present state of our knowledge of creatine metabolism the above results are difficult of interpretation. The hypothesis that the ease with which creatinuria can be induced in normal women may be due to their relative lack of muscular development (as compared with that of men) immediately presents itself, but the results recorded above cannot be interpreted as furnishing evidence in favor of such a theory.

SUMMARY.

In two normal women it was found possible to cause creatine excretion by feeding a high protein (creatine-free) diet, and to

trace of diacetic acid was found on the 3rd day and a larger amount (about 10 mg. calculated as acetone) on the 4th day. In the case of Subject II traces of acetone bodies appeared only on the 4th day.

cause the creatinuria so produced to disappear by the consumption of a low protein diet. In two men to whom a similar experimental procedure was applied we were unable to produce creatine excretion even when a sufficient amount of protein was consumed to cause the urinary nitrogen to rise to 34.5 gm. per day.

EXPERIMENTAL STUDIES ON GROWTH.

IX. THE INFLUENCE OF TETHELIN UPON THE EARLY GROWTH OF THE WHITE MOUSE.

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(Received for publication, July 19, 1917.)

Object of the Experiments.

Previous investigations have shown[1] that the administration of tethelin, a lipoid extracted from the anterior lobe of the pituitary body,[2] leads to marked retardation of the growth of the white mouse during the first 10 weeks of the third or "adolescent" growth cycle, a retardation which is succeeded by pronounced acceleration. The effect of these phenomena is to completely distort the form of the curve of growth in the third growth cycle, giving the appearance of great prolongation and enlargement of the second growth cycle (which normally terminates at 5 weeks,[3]) and an acceleration and curtailment of the third growth cycle.

In the experiments referred to, the administration of tethelin was initiated when the animals were 5 weeks old and the second growth cycle had already begun to merge into the early stages of the third. The observed effects are therefore to be interpreted as effects of tethelin upon the third or final cycle of growth during which maturity of the sexual organs is attained, together with possible effects upon the residual growth still attainable by a continuation of the second growth cycle. It appeared of considerable importance to supplement these results by ascertaining the effects of tethelin upon the first (infantile) and second growth

[1] Robertson, R. B., *J. Biol. Chem.*, 1916, xxiv, 397. Robertson, T. B., and Burnett, T. C., *J. Exp. Med.*, 1915, xxi, 280.

[2] Robertson, *J. Biol. Chem.*, 1916, xxiv, 409; *Endocrinology*, 1917, i, 24.

[3] Robertson, *J. Biol. Chem.*, 1916, xxiv, 363.

cycles of the white mouse. The following experiments were accordingly undertaken.

Methods.

The method employed was similar to that utilized by Robertson and Cutler[1] in determining the effects of lecithin and cholesterol upon the growth of suckling mice. Since, during the first 3 weeks of extra-uterine development, mice are dependent upon the mother for their nutrition, administration of substances directly to the young by mouth is attended with difficulties, while the manipulations incident to hypodermic or intraperitoneal administration might very conceivably exert an effect upon the welfare and thus indirectly upon the growth of the animals which might mask or distort the effects which are the object of inquiry. We have therefore sought to influence the growth of the young during the period of lactation by administration of the tethelin by mouth to the mother, fully recognizing, however, that the interpretation of the results obtained during this period is complicated by the possibility that the quantity and quality of the mother's milk may be somewhat affected by the administration and also by the possibility that the tethelin may be utilized or destroyed by the tissues or in the mammary glands of the mother so that it may fail altogether to reach the young. The decided effects upon the growth of suckling mice obtained by Robertson and Cutler as a result of administering cholesterol to the mother, however, encouraged us to adopt this method in the present investigation.

During the period of growth subsequent to weaning (3rd to 5th weeks) and comprising the greater part of the second growth cycle the mother was removed from the cage containing the young which thereafter received the tethelin directly with their food. The administration of tethelin was discontinued at the end of the 5th week (*i.e.*, on the 35th day after birth) but the animals, thereafter fed upon a normal diet, were weighed on the 42nd and 49th days after birth in order to determine the residual or continued effects of the tethelin previously administered.

Sixty litters of mice were divided into three groups, A, B, and C. The division was made at the birth of the litters, the litters

[1] Robertson, T. B., and Cutler, E., *J. Biol. Chem.*, 1916, xxv, 663.

being alternated so that the first litter came in group A, the second in group B, the third in Group C, and so on. In this way the groups obtained consisted of nearly equal numbers of initially similar animals. The litters were kept in separate cages during the period of the experiment and the mother was supplied with an abundance of rolled barley and water and occasionally with fresh lettuce leaves and dried bread. In addition to this the control animals (Group A) each received daily 1 cc. of mixed yolk and white of egg, another group of animals (B) received the same amount of egg mixture to which, however, were added 10 mg. of tethelin dissolved in 0.2 cc. of distilled water, while the third group (C) received the same amount of egg mixture daily to which, after 14 days, *i.e.*, after the termination of the first or infantile growth cycle, were added 10 mg. of tethelin dissolved in 0.2 cc. of distilled water. The animals were weighed daily (with occasional omissions of 1 day) to the nearest cg., each mouse in all the litters being weighed separately. The litters were all kept in the same room and under identical conditions.

The dosage of tethelin was not modified with the growth of the animals. The dosage per gm. of body weight therefore diminished very rapidly with age. Excluding the uncertain proportion, very possibly, as we shall see, amounting to the whole of the substance administered, which may have been destroyed or appropriated by the tissues of the mother during the 21 days of lactation, the average dosages per gm. of the young amounted approximately to the following.

Age.	Approximate dosage per gm. of young.
days	*mg.*
0	1.33
7	0.67
14	0.52
21	0.40
28	0.31
35	0.26

This may be compared with the dosage, varying between 0.30 mg. at 5 weeks and 0.15 mg. at 1 year, administered to the mice employed in the experiments previously reported.

DISCUSSION.

The results obtained are shown in Table I and depicted graphically in Fig. 1, in which the continuous curve denotes the growth curve of the normal animals, the broken curve that of the animals which received tethelin from birth to 35 days, and the dotted curve that of the animals which received tethelin from 14 to 35

FIG. 1. The influence of tethelin upon the early growth of white mice.

days of age. It will be seen that prior to 14 days of age the administration of tethelin to the mother was absolutely devoid of effect upon the growth of the young, the two curves of growth being so nearly identical as to be indistinguishable from one an-

TABLE I.

Age.	A. Normal.		B. Tethelin.		C. Tethelin after 14th day.	
	No. weighed.	Average weight.	No. weighed.	Average weight.	No. weighed.	Average weight.
days		*gm.*		*gm.*		*gm.*
0	118	1.47	88	1.61	126	1.47
1	111	1.73	102	1.82	107	1.67
2	116	1.96	103	2.01	106	1.85
3	100	2.28	81	2.31	99	2.23
4	84	2.53	90	2.51	108	2.47
5	78	2.76	81	2.85	89	2.76
6	79	3.06	60	3.14	60	3.02
7	91	3.35	82	3.35	100	3.27
8	80	3.66	83	3.59	85	3.54
9	91	3.86	83	3.76	85	3.72
10	82	4.11	64	4.04	79	4.09
11	73	4.36	75	4.15	82	4.29
12	67	4.25	74	4.44	77	4.39
13	65	4.53	55	4.72	49	4.75
14	82	4.44	78	4.64	87	4.74
15	75	4.74	65	4.79	76	4.93
16	87	4.81	69	4.85	77	4.94
17	76	5.12	52	5.39	76	5.33
18	64	5.16	63	5.44	74	5.55
19	60	5.28	63	5.83	77	5.65
20	65	5.99	54	6.26	55	6.09
21	74	5.89	70	6.64	83	6.51
22	67	6.74	65	6.92	75	7.05
23	79	7.03	63	7.19	79	7.38
24	66	7.52	51	8.00	76	7.86
25	63	7.91	66	8.06	71	8.36
26	63	8.00	61	8.67	73	8.23
27	62	8.65	48	9.02	49	8.84
28	74	8.55	63	8.88	78	9.30
29	65	9.49	61	9.27	63	10.02
30	73	9.62	66	9.60	60	10.20
31	67	10.04	46	10.36	66	10.41
32	57	10.52	50	10.91	70	10.98
33	54	10.67	56	11.02	64	11.10
34	52	11.14	43	11.71	43	11.71
35	65	11.08	57	11.61	73	11.77
42	140	14.85	52	13.91	69	13.83
49	80	17.43	42	15.90	55	15.59

other in the diagram. After 14 days a noticeable acceleration of growth occurs, and that this acceleration is a genuine effect of the administration of tethelin is indicated by the fact that the growth curves of the two tethelin-fed groups overlie one another so as to be almost indistinguishable from one another in the diagram, while the normal (continuous) curve diverges from them as indicated in the figure. This acceleration persists until the culmination of the second and initiation of the third cycle after which a decisive retardation, as observed in the previously reported experiments, occurs. This retardation is exhibited notwithstanding the cessation of the administration of tethelin at 5 weeks, corresponding with the conclusion of the second cycle.

TABLE II.

Variability.

Age.	A. Normal.	B. Tethelin.	C. Tethelin after 14th day.
days	*per cent*	*per cent*	*per cent*
0	18.4	17.7	21.2
7	20.8	17.5	21.8
14	20.3	20.6	22.2
21	28.7	20.2	27.1
28	26.2	26.1	25.0
35	26.2	23.2	22.7
42	20.5	21.6	20.1
49	16.0	18.5	16.8

The effects of the administration therefore persist for a considerable period after its discontinuance.

The variabilities of the weights of the three groups of animals at 7 day intervals computed in the manner described in previous communications,[5] are enumerated in Table II. No decisive effect of the administration upon the variability of the animals is observable, although the animals receiving tethelin display a slight tendency to diminished variability, the mean variability of the normal group during the period from and including 21 to 49 days being 23.5 per cent while the mean variabilities of the tethelin-fed groups during the same period were 21.9 and 22.3 per cent.

[5] Robertson, *J. Biol. Chem.*, 1916, xxiv, 363, 385, 397; *Am. J. Physiol.*, 1916, xli, 535.

No effect was observed of the administration of tethelin upon the date at which the eyes of the young opened, which is a very sharply defined criterion of development. Since the animals of Group C did not receive tethelin until the 14th day, the animals of both Groups A and C serve during the first 14 days of development as controls with which the development of Group B may be compared. It will be observed (Table III) that the average date at which the eyes opened in the young of group B is intermediate between the dates at which the eyes opened in the young of the two control groups. In view of the absence of effect upon growth in weight during the first 14 days, the lack of effect of administration of tethelin to the mother upon structural development as evidenced by the opening of the eyes was to be anticipated.

TABLE III.

Opening of Eyes.

Class.	Average age at which eyes opened.	Variability of age at which eyes opened.
	days	*per cent*
A (Normal)	14.4	7
B (Tethelin)	14.0	6
C (Tethelin after 14th day)	13.6	9
A + C	13.8	8

The comparative invariability of the period at which the opening of the eyes occurs has been commented upon elsewhere.[3, 6] As will be observed on comparing the figures enumerated in Tables II and III the variability of the period at which the eyes open is correlated with the initial variability in weight of the young at birth, the least variable group in weight at birth displaying the least variable period at which the eyes open, while the most variable group in weight at birth (C) displays the most variable period at which the eyes open. Hence the attainment of this stage of development, although relatively so invariable a phenomenon, is nevertheless in some measure affected by the much more variable phenomenon of growth in weight.

[6] Daniel, J. F., *Am. Naturalist*, 1912, xlvi, 591.

SUMMARY.

From the total lack of effect of the administration of tethelin to the mother upon the growth of suckling young we may infer either that tethelin exerts no effect upon growth during the first (infantile) growth cycle or else, which is more probable, that the tethelin administered to the mother is not secreted as such to any appreciable extent by the mammary glands of the mother.

The administration of tethelin to the young subsequently to the 14th day, when their eyes are open and they have access to food other than that supplied to the mother, results in a noticeable acceleration of growth during the second growth cycle (2nd to 5th weeks), followed, upon initiation of the third cycle, by a marked retardation which evidences itself despite the fact that the administration of tethelin is discontinued at the end of the 5th week.

Variability of the period at which the eyes open is correlated with the initial variability in weight of the young at birth.

THE BLOOD LIPOIDS IN NEPHRITIS.

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(Received for publication, July 28, 1917.)

At the time when bleeding was a common therapeutic practice nephritis was one of the conditions in which milkiness of the plasma was occasionally observed (1), and blood examinations in recent years give support to these earlier reports—indicating that there may be a disturbance of fat metabolism in this disease.

Thus Watjoff (2) found, in a case of nephritis, microscopically visible fat which stained with osmic acid. Bönniger (3) reported blood fat (total lipoids) high. Erben's (4) analyses showed increased values for fat and lecithin in a subchronic case. Greenwald (5) found high lipoid phosphorus in some of his series of nephritics. Chauffard, La Roche, and Grigaut (6) reported hypercholesterolemia in chronic nephritis with lipemia (milky plasma) in a case of uremia, and Widal, Weill, and Laudat (7) found lipemia frequently in nephritis. Henes (8) observed that the blood cholesterol was increased and that the increase was greatest in the most severe cases. He called attention, however, to the fact that in one fatal case with uremia the cholesterol value sank shortly before death. J. Müller (9) gave the following high values for the blood lipoids in a case of nephritic lipemia (blood taken post mortem): Total ether extract 3.6 per cent; neutral fat 2.15; cholesterol 0.84; lecithin 0.69. Schmidt (15) found in most patients with hypertension, in whom kidney function was not far from normal, that the cholesterol values were high, while if marked functional deficiency existed the values were normal or below. Epstein and Rothschild (10) found in chronic parenchymatous nephritis, particularly in the edematous stage, that the blood lipoids were very high—cholesterol up to 1.23 gm. per 100 cc. of blood, while in uremic cases, especially those with high nitrogen retention, the lipoids were much diminished—cholesterol as low as 0.08 gm. per 100 cc. The origin of the high lipoid they believed to be ingested or mobilized fat representing a condition of non-utilization, as evidenced by the fact that on a low fat diet the lipemia disappeared. Denis (11) found a notable increase in blood cholesterol in nephritis in only one case out of about fifty of various types examined. She suggests that the lack of high values in her series may have been due to a diet which contained little cholesterol.

[illegible]

Methods.

[illegible]

[illegible]

directly into a pipette by means of a needle and short piece of rubber tubing, and run from the pipette into the centrifuge tube, or if the lipoid values of the whole blood only are desired the blood may be run directly from the 3 cc. pipette into the alcohol-ether.

It is desirable to get the blood into the alcohol-ether as soon as possible after drawing so as to avoid possible changes in the lipoids by standing. After it is once precipitated it may be allowed to stand in the stoppered flasks for a week or more until a number of samples have collected or until it is convenient to proceed to the next step. Values do not appear to be affected in any way by this step. The filtered extracts when kept in tightly stoppered bottles in a cool place in the dark have been found to keep unchanged for at least 6 months so that the procedure offers a convenient way of collecting samples and storing them until it is convenient to make the analyses.

Total Fat.—In the determination of total fat the following changes have been made. To the blood extract after saponification are added 5 cc. of alcohol-ether, and the whole is raised to boiling. The liquid is then removed from the heat and after active bubbling has ceased the ether vapor is blown off, the beaker meanwhile being gently shaken. The 5 cc. of standard solution are heated in the same way (a few grains of coarse sand are added to promote even boiling). This treatment removes most of the ether, which if allowed to remain tends to produce bubbles in the water solution and also to cause differences in color (brownish tints) in one or other solution, due to slight differences in the aggregation of the precipitated material. Both standard and test solutions are thus treated throughout in as nearly the same way as possible so that the changes in volume produced by the loss of the ether are the same in each. To each of standard and test solutions are now added 50 cc. of distilled water, the solutions well stirred to ensure complete solution of the soaps, and the readings made according to the original directions. If the drying of the saponification mixture has been carried too far or if the temperature at the end of the drying has been too high, low values will be obtained, due probably either to baking of the mixture on the bottom of the beaker or to partial destruction of the cholesterol by the hot concentrated alkali. For this

Blood Lipoids in Nephritis.

	Corpuscles.	Total fatty acids. Whole blood.	Total fatty acids. Plasma.	Total fatty acids. Corpuscles.	Lecithin. Whole blood.	Lecithin. Plasma.	Lecithin. Corpuscles.	Cholesterol. Whole blood.	Cholesterol. Plasma.	Cholesterol. Corpuscles.	Fat. Plasma.	Fat. Corpuscles.	Total fatty acids / Lecithin. Whole blood.	Total fatty acids / Lecithin. Plasma.	Total fatty acids / Lecithin. Corpuscles.	Lecithin / Cholesterol. Whole blood.	Lecithin / Cholesterol. Plasma.	Lecithin / Cholesterol. Corpuscles.	Plasma total lipoid.	Kidney function.*	Non-protein nitrogen.*	Remarks.
	per cent																			*per cent*	*mg.*	
Normal average.		0.36	0.39	0.32	0.30	0.20	0.42	0.22	0.23	0.20	0.13	0.04	1.25	1.90	0.79	1.35	0.9	2.1	0.7			
1 S 220	45	0.42	0.33	0.53	0.32	0.18	0.50	0.16	0.14	0.18	0.12	0.18	1.30	1.83	1.06	2.0	1.3	2.8	0.50	60	43	
2 O'B 206	43	0.54	0.49	0.60	0.29	0.16	0.46	0.18	0.18	0.18	0.30	0.14	1.8	3.0	1.3	1.6	0.9	2.6	0.71	55	32	
3 S 207		0.38			0.14			0.15					2.7			0.9				40	30	
4 R 224		0.48			0.29			0.17					1.6			1.7				45	71	
5 J 200	35	0.39	0.45	0.30	0.26	0.14	0.48	0.19	0.17	0.23	0.29	0.0	1.5	3.2	0.6	1.4	0.8	2.1	0.67	30	40	Acute glomerular nephritis.
6 O 227	33	0.41	0.40	0.45	0.25	0.14	0.47	0.19	0.18	0.20	0.23	0.12	1.6	2.9	0.95	1.3	0.8	2.4	0.62	20	51	Dyspnea.
7 R 218	37	0.48	0.48	0.48	0.37	0.27	0.57	0.22	0.19	0.27	0.22	0.08	1.3	1.8	0.8	1.7	1.4	2.1	0.76	20	58	
8 B 201	34	0.46	0.52	0.34	0.26	0.15	0.47	0.25	0.22	0.30	0.32	0.0	1.8	3.5	0.7	1.0	0.7	1.6	0.76	20	107	
9 I 225	33	0.55	0.40	0.86	0.32	0.14	0.70	0.18	0.16	0.25	0.24	0.37	1.7	2.8	1.2	1.8	0.9	2.8	0.60	15	65	Dyspnea.
10 H 203	25	0.53	0.59	0.35	0.31	0.24	0.52	0.28	0.30	0.22	0.30	0.6	1.7	2.5	0.67	1.0	0.8	2.4	0.96	10	47	
11 K 202	19	0.57	0.56	0.60	0.27	0.18	0.65	0.19	0.18	0.23	0.34	0.14	2.1	3.1	0.9	1.4	1.0	2.8	0.77	10	107	Acute nephritis.
12 H 217	34	0.42	0.47	0.34	0.40	0.26	0.55	0.22	0.24	0.19	0.24	0.0	1.1	1.7	0.6	1.8	1.1	2.9	0.84	10	55	Uremia.
13 P 213	40	0.40	0.30	0.47	0.25	0.14	0.42	0.16	0.16	0.16	0.14	0.18	1.6	2.1	1.1	1.6	0.9	2.6	0.50			Acute nephritis, pneumonia.
14 S 212	20	0.64	0.66	0.56	0.21	0.19	0.29	0.20	0.18	0.28	0.16	0.35	3.0	3.3	1.9	1.1	1.0	1.0	0.90			

15 E 223		0.47			0.33			0.19					1.4			1.7				4	45	Acute nephritis, edema.
16 M 221	32	0.53	0.53	0.53	0.34	0.27	0.49	0.22	0.22	0.22	0.26	0.18	1.6	2.0	1.1	1.5	1.2	2.2	0.84	5	111	Very dyspneic.
17 " 222	24	0.63	0.67	0.50	0.36	0.24	0.74	0.21	0.21	0.21	0.42	0.0	1.8	2.8	0.7	1.7	1.1	3.5	0.95			Coma, death.
19 M 205	36	0.57	0.64	0.45	0.32	0.16	0.60	0.22	0.17	0.30	0.45	0.0	1.8	4.0	0.7	1.5	0.9	2.0	0.85	3	69	Marked acidosis, death.
20 G		0.60			0.26			0.21					2.3			1.2						Coma, death.
21 H 228	39	0.59	0.62	0.54	0.29	0.23	0.39	0.21	0.23	0.17	0.37	0.26	2.0	2.7	1.4	1.2	1.0	2.3	0.92		89+	Coma and death 1 week later.
22 O'F 219	14	0.49	0.47	0.61	0.30	0.25	0.60	0.16	0.15	0.17	0.23	0.19	1.6	1.9	1.0	1.9	1.7	3.5	0.69	0	232	Blood pressure 205, coma, and death.
23 C 204	13	0.63	0.63	0.63	0.25	0.18	0.70	0.19	0.18	0.25	0.42	0.13	2.5	3.5	0.9	1.3	1.0	2.8	0.85	0	187	Uremia, death, phosphates increased. Blood sugar 0.22.

* These values were obtained from the records of the Massachusetts General Hospital.

reason the drying should be done on a water bath rather than on an electric stove. The use of 50 cc. of water instead of the original 100 cc. gives a solution which is more easily read and when most of the ether is removed as above there is no trouble with bubbles.

Cholesterol.—Very few changes have been made in the method as described. A gentle air blast is used to hasten evaporation of the blood extract and to prevent over-heating, which produces a brownish tint and destroys part of the cholesterol. The directions for the production of the color have been slightly modified. Because of the high price of satisfactory acetic anhydride the amount used has been reduced from 2 cc. to 1 cc. for each determination. The smaller amount has been found entirely adequate for the amounts of cholesterol determined. The acetic anhydride obtainable at the present time is frequently of poor quality, either colored or developing a color in the determination. Distillation has been found to improve it. The temperature at which the color is produced is within a degree or so of 22°C., rather higher than lower. This temperature is the ordinary room temperature in most laboratories.

Lecithin.—The strychnine molybdate precipitation is now used altogether instead of the less convenient and less sensitive silver precipitation. The directions for the use of this reagent in the determination of lecithin have been given previously (13). A dilute sulfuric acid (one part of concentrated acid with three parts of water) is used for neutralization instead of the concentrated acid which was originally recommended because of the necessity with the silver precipitation of keeping the volume of solution small. A number of determinations indicate that, with the molybdate precipitation, the use of cane sugar solution and second heating recommended in the earlier directions is probably not necessary and it has been omitted in the later determinations.

The results of the determinations of the blood lipoids are given in Table I, the values being expressed in gm. per 100 cc. of blood.

The cases are arranged in the table in approximately the order of their severity and, for completeness, the values for non-protein nitrogen (in mg. per 100 cc. of blood) and kidney function (as determined by per cent excretion of phenolsulfophthalein), obtained from the records of the Massachusetts General Hospital, are included.

RESULTS AND DISCUSSION.

Total Fatty Acids.—High in both plasma and corpuscles.

Lecithin.—In the plasma lecithin was generally normal or below while in the corpuscles it was frequently high and especially so in Cases 222, 205, 204, and 219, where the blood samples were taken shortly before death.

Cholesterol.—Practically normal throughout.

Fat.—In the plasma this value was, with two exceptions, much above the normal. In the corpuscles it was frequently abnormally high and the high values were most marked in those cases with the most severe symptoms.

Total Fatty Acids: Lecithin.—In the plasma this ratio was generally much above normal (due to excess of fat). In the corpuscles it was occasionally high.

Lecithin: Cholesterol.—This ratio was normal in most cases in both plasma and corpuscles but high in those cases with more severe symptoms.

Total Lipoids.—Generally above normal.

The percentage of corpuscles was generally below normal and in some of the severe cases very much below, as in the last two of the series.

The plasma in this series of cases was free from visible fat although occasionally muddy with material in coarse suspension.

The most marked abnormalities observed in the blood lipoids in this series were then as follows:

1. High values for total fatty acids in both plasma and corpuscles.

2. High fat in the plasma (with occasional high values in the corpuscles) which in general was most marked in those cases with more severe symptoms.

3. Frequent high values for lecithin in the corpuscles, which were very marked in fatal cases where the samples were taken shortly before death.

4. High values for total lipoids in the plasma.

The high total fatty acids in the plasma and corpuscles and the high lecithin in the corpuscles with normal cholesterol are the conditions observed in the blood in alimentary lipemia (13) and

therefore strongly suggest that the abnormalities observed in the lipoids in nephritis are due to a retarded fat assimilation in the blood. The extent of the abnormality would in that case depend on at least three factors—the extent of the retardation, the amount of fat present in the blood when the process began, and the amount of fat entering the blood from the alimentary canal. The great variations in the blood lipoids in nephritis reported in the literature are explainable as the result of differences in these factors.

On the basis of work already done on the blood lipoids it seems possible to distinguish between "acute" and "chronic" disturbances in the blood lipoids—acute disturbances such as occur in alimentary lipemia, which are characterized by increased fat and lecithin, and chronic disturbances characterized also by increased cholesterol, of which the best example is diabetes. The excess of lipoids in both these examples frequently results in milkiness of the plasma. The abnormalities in nephritis observed in this work would, on this basis, be classified as acute disturbances, but there is some evidence in the literature to show that the condition may become chronic with high cholesterol values. The best example of this chronic condition reported is that of Müller already noted (9). In this case the distribution of the blood lipoids was similar to that found in diabetes—high fat (glycerides) with cholesterol increased almost parallel with the fat, while lecithin, although much above the normal value, had still not increased to anything like the extent of the other two. The plasma in this case was milky. Other cases reported in the literature had high cholesterol and lipemia (6, 7). No milkiness of the plasma was observed in any of the series reported in this paper although the plasma was occasionally muddy from matter in coarse suspension, which was probably not fat.

As to the cause which produces these disturbances in the lipoids in nephritis, the most frequent other condition in which abnormalities of the blood lipoids are common is diabetes and a prominent symptom of severe diabetes is "acidosis"—a decreased "alkali reserve" in the blood. Recent investigations (14) have established the fact that acidosis is frequently a feature of severe nephritis, and in most of the cases of this series acidosis was probably present, as evidenced by the dyspnea, low carbon dioxide

tension in the alveolar air, and coma. Since an adequate alkalinity of the blood and tissues is necessary for their normal functioning it seems very probable that the retardation of fat assimilation found in nephritis is one manifestation of a general phenomenon brought about by a decreased blood and tissue alkalinity.

SUMMARY.

The abnormalities in the blood lipoids in severe nephritis were found to be high fat in plasma and corpuscles and high lecithin in the corpuscles. The cholesterol values were practically normal. These abnormalities are the same as are found in alimentary lipemia and for this reason are regarded as the result of a retarded assimilation of fat in the blood, which in turn is thought to be one manifestation of a general metabolic disturbance brought about by a lowered "alkali reserve" of the blood and tissues.

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THE DYNAMICS OF THE PROCESS OF DEATH.

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The writer has found that by measuring the electrical conductivity of tissues placed in toxic solutions the process of death can be followed in the same manner as the progress of a reaction *in vitro*.

Studies on a considerable variety of toxic solutions have shown that in them death proceeds as a monomolecular reaction. In the case of *Laminaria* in NaCl, it was observed[1] that the reaction behaves as if it were "inhibited" at the start, as shown by the fact that the velocity constant was fairly regular except at the start, where it was clearly below the average value. At that time the writer was unable to suggest a satisfactory explanation of this "inhibition." Later studies[2] have afforded a clue to the explanation. In these studies it was found necessary to assume that death should be regarded as a series of reactions of the type $O \rightarrow A \rightarrow M \rightarrow B$.

It is assumed that M is a substance which determines the normal permeability and electrical resistance (and perhaps other normal properties) of the protoplasm. As long as the tissue remains in its normal environment M is formed as rapidly as it is decomposed but when the tissue is placed in NaCl (which is toxic for *Laminaria*) the reaction $O \rightarrow A$ ceases, while the reactions $A \rightarrow M \rightarrow B$ proceed at an increased rate. As a result the quantity of M decreases to zero, at which point the tissue is regarded as dead. This point can be determined by measuring the electrical conductivity of the tissue as can any intermediate point (*e. g.*, half dead, etc.).

[1] The methods of measurement and of calculation are explained in *Science*, 1914, xxxix, 544.

[2] Osterhout, W. J. V., *Proc. Am. Phil. Soc.*, 1916, lv, 533.

We may assume for convenience that in sea water (the normal environment of *Laminaria*) the concentrations[3] of A and M are constant at 8.853 and 0.2951 respectively, and that on transferring to NaCl the velocity of the reactions $A \rightarrow M$, and $M \rightarrow B$, increases to 0.018 and 0.540 respectively. We assume that these reactions are monomolecular and irreversible (or practically so). We can then calculate the amount of M (this amount will for convenience be called y) at any time, T, after the tissue is transferred to the solution of NaCl.

As explained in a previous paper,[2] we may employ for this purpose the formula:

$$y = 0.2951\,(e^{-K_2T}) + 8.853\left(\frac{K_1}{K_2 - K_1}\right)(e^{-K_1T} - e^{-K_2T})$$

in which y is the amount of M, T is the time of exposure to the solution of NaCl, e is the basis of natural logarithms, and K_1 and K_2 are the velocity constants of $A \rightarrow M$ and $M \rightarrow B$ respectively.

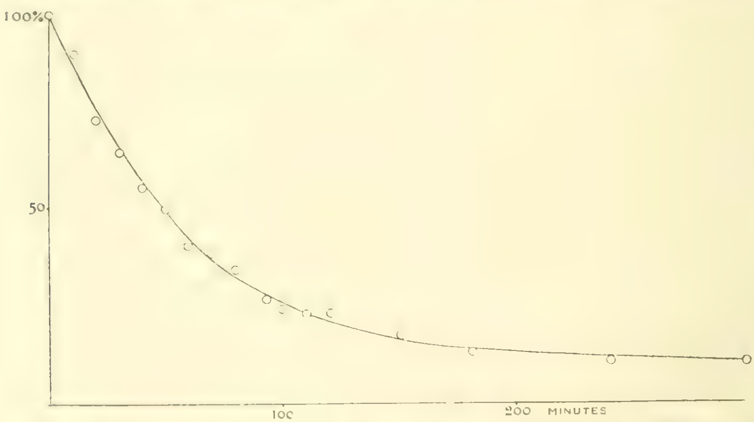

FIG. 1. Electrical resistance of *Laminaria* in a solution of NaCl. Experimental values (o, o) and calculated curve.

The results of a series of such calculations are given in Table I, together with the values obtained in a recent series of experiments. The calculated and observed values are plotted in Fig. 1. It will be seen that the agreement is very satisfactory.

[3] These values were used because they had been employed in previous calculations. The values of y are multiplied by 305 and 10 is added.

TABLE I.*

Net Electrical Resistance of Laminaria in NaCl 52 M. The Resistance in Sea Water (the Normal Environment) Is Taken as 100 per Cent.

Time.	Resistance.		K_3	
	Observed.	Calculated.	From observed values.	From calculated values.
min.	*per cent*	*per cent*		
10	87.50	87.76	0.0065	0.0064
20	73.01	74.96	0.0077	0.0071
30	62.51	64.26	0.0078	0.0073
40	55.30	55.32	0.0075	0.0075
50	48.81	47.86	0.0073	0.0075
60	40.21	41.62	0.0079	0.0076
70	36.79	36.41	0.0075	0.0076
80	32.41	32.06	0.0076	0.0076
90	27.52	28.43	0.0079	0.0077
100	24.69	25.39	0.0079	0.0077
110	23.00	22.86	0.0076	0.0077
120	22.82	20.74	0.0071	0.0077
150	16.51	16.26	0.0076	0.0077
180	14.54	13.65	0.0072	0.0077
Average.................................			0.0075	0.0075

* All readings were made at 15°C., or corrected to this temperature.

If we were unaware that this curve represented two consecutive reactions, and supposed it to represent a simple monomolecular reaction ($M \rightarrow B$), we should calculate its velocity constant by the usual formula:[4]

$$K_3 = \frac{1}{T} \log \left(\frac{a}{a - x}\right)$$

If we make this calculation, employing for this purpose the calculated values given in the third column of Table I, we obtain the values of the velocity constant K_3 given in the fifth column of Table I.

It is evident from an inspection of these values that the velocity constant K_3 falls below the average value at the start.

The amount by which it falls below the average value will depend on the relation $K_1 \div K_2$. When K_1 and K_2 are nearly

[4] Common logarithms are used for convenience.[1] We put $a = 100 - 10$ and $a - x = y - 10$.

equal, the velocity constant falls a good deal below the average value at the start, but as the difference between them is increased the velocity constant K_3 will be found to fall less and less below the average value at the start.[5] This is easily shown by assuming various values[6] of K_1 and K_2.

From this it follows that we can tell something about $K_1 \div K_2$ from the experimental values of K_3. It is evident that in the present case the experimental values of K point to the relation $K_2 \div K_1 = 30$ (or $K_1 \div K_2 = 30$). This relation was actually assumed by the writer in a previous paper in order to fit, not the NaCl curve, but antagonism curves[7] in various mixtures of NaCl + $CaCl_2$. It is therefore a striking confirmation of the general correctness of the underlying assumption that we are also able by this assumption to fit the NaCl curve so closely.

In general, where a chemical reaction is slower at the start than is expected, we may suspect that we have to do, not with a simple reaction, but with consecutive reactions of the kind here described.[8]

This explanation also applies to a considerable number of other cases of toxic action.

It is of interest that in all these cases death behaves as a reaction which is continually going on but at a very slow rate until accelerated by the toxic agent. We have assumed this acceleration to consist partly in the increase of the velocity constant and partly in the stopping of the reaction $O \rightarrow A$, causing a decrease in the substance (M) to which normal permeability (and perhaps other normal properties) are due.

It may prove to be generally true that death behaves as a monomolecular reaction, more or less inhibited (or accelerated)

[5] It should be noted that we get the same result (as regards K_3 falling below the average at the start) when $K_1 \div K_2 = 30$ as when $K_2 \div K_1 = 30$. With certain relations of $K_1 \div K_2$ the constant K_3 may be above the average value at the start.

[6] When the values of K_1 and K_2 are changed, the concentrations of A and M must also be changed in such a way that Conc. $A \div$ Conc. $M = K_1 \div K_2$ if we wish the concentrations of A and M to remain constant in the normal environment.

[7] The manner in which the relation $K_1 \div K_2$ influences the forms of these curves cannot be discussed in this paper.

[8] Mellor, J. W., Chemical Statics and Dynamics, London, 1909, chapt. vi.

at the start. The assumption of consecutive reactions affords an explanation not only of the inhibition (or acceleration) at the start but also of the fact that up to a certain point the reaction appears to be reversible. The latter fact will be fully discussed in a subsequent paper.

SUMMARY.

In the case here described, death proceeds as a monomolecular reaction which is somewhat "inhibited" at the start. This is easily explained if consecutive reactions are involved in the process of death. This explanation also applies to many other cases of toxic action.

In all these cases death behaves as a reaction which is continually going on and which is accelerated by the toxic agent.

THE STRUCTURE OF YEAST NUCLEIC ACID.

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(Received for publication, July 26, 1917.)

Levene and Jacobs have formulated the structure of yeast nucleic acid as a tetranucleotide. The facts that led up to their formulation were:

1. The formation of four nucleosides on neutral or ammonia hydrolysis.

2. The formation of simpler nucleotides on hydrolysis with dilute mineral acids.

3. The presence of a phosphorus to nitrogen ratio which agreed quite well for the tetranucleotide theory.

4. The ratio of amino to total nitrogen in nucleic acid was in harmony with the ratio required by the four bases, guanine, adenine, uracil, and cytosine.

The mode of linkage between the individual nucleotides was at that time not determined, and in the graphic formula representing the nucleic acid the linkage of the nucleotides was of a provisional character and arbitrary in nature.

Following that, the studies of Levene and Jacobs on thymus nucleic acid led to methods which permitted the separation and the study of individual mono- and dinucleotides composing the complex nucleic acids. Levene and Jacobs then returned to the study of yeast nucleic acid, applying the experience gained on the thymus nucleic acids. They then directed their attention to the nucleotides obtained by acid hydrolysis of the yeast nucleic acid. A large quantity of the material was prepared and transformed into the brucine salt. Other work, however, made a demand on their energies, and the work on the nucleotides was somewhat neglected. However, in course of the present academic year the work has been resumed. There were on hand 125.0 gm. of the brucine salts when the work was begun.

Meanwhile Dr. Walter Jones and his coworkers published several important publications on the structure of yeast nucleic acid. The basis of their work is the conception of the nucleic acid molecule as expressed by Levene and Jacobs.

The work of Jones deals specifically with the mode of linkage between individual nucleotides; Jones accepts a tetraribose of the structure ($(C_5H_{10}O_5)_4-3H_2O$) as the nucleus of the molecule. In this nucleus all the carbonyl groups of course must be free, since this is demanded by the existence of nucleosides. Dr. Jones bases his conclusion on two arguments: one is a proof by analogy; namely, the wide distribution of polysaccharides in nature; the other is the discovery by him of dinucleotides in which four of the hydroxyls of the phosphoric acid are free. The first argument does not seem valid, since according to present knowledge all the polysaccharides in nature have a glucosidic structure, and the one assumed by Jones to be present in the molecule of nucleic acid can be constructed only through ether linkage. Just because a proof by analogy is lacking, all the greater rigor is required from the experimental evidence.

There have appeared three papers by Jones and his coworkers dealing with the subject. In one, Jones and Richards claim to have cleaved the molecule of yeast nucleic acid into two large fractions, guanine-cytosine dinucleotide, and adenine-uracil dinucleotide. However, the authors admit that the dinucleotides were not isolated in pure form.

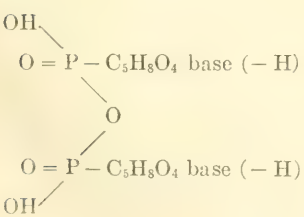
In a second paper Jones and Germann claim to have accomplished by ammonia hydrolysis the same cleavage as Jones and Richards had accomplished by enzymes. However, from the fraction named guanosine-cytosine only guanylic acid was obtained. The adenine-cytosine fraction analyzed for the dinucleotide and a brucine salt obtained from it analyzed satisfactorily for the same substance. The uracil nucleotide was not traced.

In a third paper Jones and Read, following closely the directions of Levene and Jacobs, prepared the pyrimidine nucleotide fraction previously obtained by these authors. Jones and Read converted it into the brucine salt and found the analytical data of this substance to agree with that of cytosine-uracil dinucleotide.

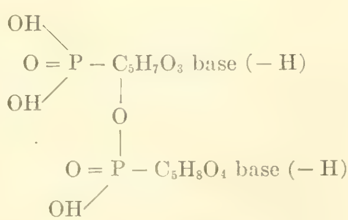
Thus the principal argument of Jones and his coworkers in favor of the dinucleotide structure of their substances is based on the analysis of the brucine salts. The alkaloid brucine was introduced into the study of nucleotides by Levene and Jacobs. The advantage of this reagent, as seen by them, consisted in the fact that it permitted fractionation of the brucine salts of nucleotides not only out of water, but also out of ethyl or methyl alcohol. The separation of the cytosine and thymine nucleotides of the thymus nucleic acid was based on this property of the brucine salts. Not much importance was attributed to the analytical data of the brucine salts. Indeed, Jones did not exaggerate the value of the analytical data of the brucine salts when he criticized the work of Tannhauser. Employing the same ammonia hydrolysis Tannhauser thought he had isolated a trinucleotide. Jones took exception to the conclusion on the basis of the fact that the percentage composition of the brucine salt was not much different from that of the brucine salt of guanylic acid.

Levene and Jacobs, in their previous work on the brucine salts of nucleotides, formulated the following requirement as a test of the individuality of a nucleotide. First, a constant composition of the brucine salt on fractional crystallization, and second, a conversion of the brucine salt into a barium salt, which furnished analytical data agreeing with the theory for the assumed substance. This seemed the minimum requirement.

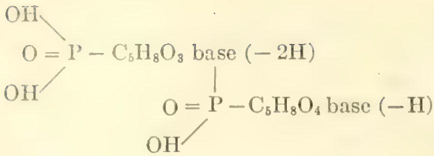
But even admitting that all the dinucleotides of Jones and coworkers actually exist, does this fact necessarily force the conclusion of a tetraribose nucleus? There are theoretically possible not only two but six ways of linkage between two nucleotides. They are:



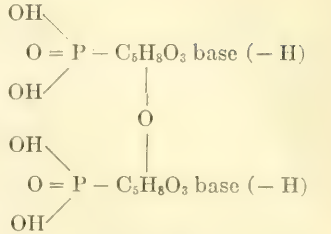
I



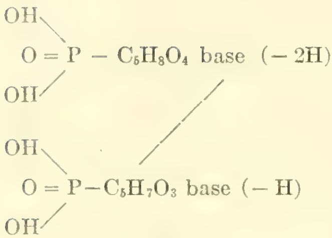
II



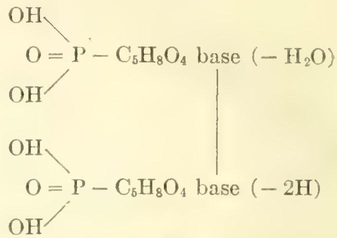
III



IV



V



VI

Forms III and V are possible of existence only when at least one of the two bases has two NH groups in the molecule and form VI when one base has two NH groups and the other, one NH and one OH groups.

In the first three forms of dinucleotides there are less than four readily ionizable hydrogen atoms. They should form di- or tribasic salts. But we have seen in guanylic acid the readiness with which a nucleotide forms a basic salt. Furthermore, dinucleotides of type II were shown to be present in thymus nucleic acid.

The remaining three may all function as tetrabasic acids. Forms IV and V may be regarded as the two most probable structures for dinucleotides forming tetrabrucine salts. A decision between these two alternatives must be based on either experimental data or on valid theoretical reasoning.

These possibilities must be particularly borne in mind, because of the two following facts: first, in purine nucleotides the phosphoric acid is less firmly linked to the nucleoside than in pyrimidine nucleotides; and second, pyrimidine nucleotides form a dinucleotide more resistant toward hydrolytic action of acids than the purine nucleotides. The latter fact was first shown by

Levene and Jacobs for thymus nucleic acid, and is now shown to hold also for yeast nucleic acid. These differences in behavior are surely due to differences in structure. If a diribose is proven to exist in all dinucleotides, then the difference will have to be explained by the differences in the position of the hydroxyl groups which form the oxidic linking of two molecules of ribose. The final decision on the details of the structure of dinucleotides has to be reserved. All these considerations make it a difficult task to express the structure of yeast nucleic acid in a graphic formula without any arbitrary elements. If such a one is desired, it can only be expressed for the present in the following manner.

$$\left[\begin{array}{l} \begin{array}{l} OH\diagdown \\ \quad O{=}P-C_4H_8O_4\ C_5H_4N_5O \\ OH\diagup \end{array} \\ \left.\left\{\begin{array}{l} OH\diagdown \\ \quad O{=}P-C_4H_8O_4\ C_4H_4N_3O \\ OH\diagup \\ OH\diagdown \\ \quad O{=}P-C_4H_8O_4\ C_4H_3N_2O_2 \\ OH\diagup \end{array}\right\} -H_2O \right. \\ \begin{array}{l} OH\diagdown \\ \quad O{=}P-C_4H_8O_4\ C_5H_4N_5 \\ OH\diagup \end{array} \end{array}\right] -2H_2O$$

In the present communication it is desired to report on the cytosine-uracil dinucleotide prepared some time ago. The crude material was prepared nearly 5 years ago and the dinucleotide was isolated about 4 months ago. The result was not communicated because more light on the mode of linkage was desired. However, because of the recent publication of Jones and Read, our results are now presented. By means of fractional extraction with methyl alcohol the crude brucine salt was separated in several fractions, each different in composition. The fractions that came closest in their composition to that of the brucine salt of the cytosine-uracil dinucleotide were converted into a crystalline barium salt. The analytical data of the substance agreed with the theory for the assumed dinucleotide. The ratio of the amino to total nitrogen of the salt agreed with the theory for the barium salt of the dinucleotide. The melting

point of our brucine salt was 200°C. (corrected). The physical constants of our substance are slightly different from those of Jones and Read. This is not surprising since Levene and Jacobs have already shown that the crude barium salt is altered in composition by reprecipitation, and it is now also shown that various fractions of the crude brucine salt vary in composition. Furthermore, we are not aware of a single instance in which a partial hydrolysis leads to products of only one phase of hydrolysis. As a rule, under such conditions, substances of different degrees of cleavage are formed. Indeed, in the partial hydrolysis of thymus nucleic acid Levene and Jacobs have shown the presence simultaneously of thymine-cytosine dinucleotides and of the mononucleotides of the same bases. Whether the substance of Jones and Read or ours is nearest to the pure dinucleotide remains to be established.

As regards the mode of linkage of this dinucleotide, structure IV may perhaps be accepted, however, not with absolute certainty, as structure V cannot be excluded without further proof. However, if structure V were to be accepted it would necessitate the acceptance of a union between a hydroxyl group of the sugar and one of the nitrogen atoms of the base. Taking into consideration the structure of the two bases uracil and cytosine,

$$\begin{array}{llll} (1) & \mathrm{HN-CO} & (6) \\ & \ \ | \qquad\ | \\ (2) & \mathrm{OC} \quad \mathrm{CH} & (5) \\ & \ \ | \qquad\ \| \\ (3) & \mathrm{HN-CH} & (4) \end{array} \quad \text{and} \quad \begin{array}{llll} (1) & \mathrm{N-CNH_2} & (6) \\ & \ \ | \qquad\ | \\ (2) & \mathrm{OC} \quad \mathrm{CH} & (5) \\ & \ \ | \qquad\ \| \\ (3) & \mathrm{HN-CH} & (4) \end{array}$$

and further, taking into consideration the fact that in cytosine the NH_2 group is unsubstituted and that therefore cytosine cannot serve as a connecting link between two nucleotides, then uracil would have to be accepted as the bridge between two riboses of the dinucleotide. Future work will have to decide between the two structures. This applies also to the pyrimidine dinucleotide from thymus nucleic acid.

EXPERIMENTAL.

The bulk of the material was the brucine salts prepared about 5 years ago from the barium salts described by Levene and Jacobs.

50.0 gm. of the brucine salt were extracted with 1,500 cc. of boiling methyl alcohol. The insoluble part was successively extracted until the insoluble residue did not perceptibly lose in weight on two successive extractions. The alcoholic extracts on standing at 25°C. formed a crystalline deposit. This was filtered, and the filtrate concentrated to dryness. The residue was recrystallized out of water. Out of 50.0 gm. there remained 14 gm. as the difficultly soluble part. This fractionation was carried out on two lots of 50.0 gm. each of the old material, and on two smaller samples prepared in course of this year. In the following table is given a summary of the analytical data of the various samples.

Sample No.	C	H	N	P	M. P.	$[\alpha]_D^{25}$
Insoluble.						
1	56.56	5.89	6.55	2.85	Contracted 178°C. Decomposed 215°C.	$\frac{-0.02 \times 10.0}{1 \times 0.05} = -4.0°$
2	56.20	6.09	6.62	2.64		
3	56.72	6.18	6.11	3.17		
Soluble.						
4	58.18	6.37	8.05	2.70	Contracted 185°C. Decomposed 200°C.	$\frac{-0.05 \times 10.0}{1 \times 0.05} = -10.0°$
5	58.99	6.68	7.89	2.79	Contracted 185°C. Decomposed 200°C.	$\frac{-0.13 \times 5.0}{1 \times 0.05} = -13.0°$
6	58.47	6.93	9.05	2.71		
Theory for dinucleotide	59.36	6.73	8.19	2.80		

Samples 5 and 6 were dissolved in warm water with the aid of a slight excess of ammonia and shaken in a separatory funnel with chloroform to remove all brucine. To the brucine-free solution barium hydroxide was added in slight excess over the quantity theoretically required to neutralize the acidity of the nucleotide, and the solution was repeatedly evaporated to dryness under diminished pressure until all ammonia was removed. The residue

was then dissolved in water and neutralized to litmus with sulfuric acid, filtered from barium sulfate, and concentrated under diminished pressure to a very small volume until an insoluble white precipitate began to form. On cooling, the precipitate increased. It was filtered, dissolved to a clear solution, and again concentrated to a small volume. This time a granular precipitate settled out which under the microscope consisted of striated plates and needles.

0.0955 gm. substance required for neutralization 4.93 cc. 0.1 N acid.
0.1909 " " gave 0.0426 gm. $Mg_2P_2O_7$.
0.0955 " " " 0.0464 " $BaSO_4$.
0.0966 " " " 0.0798 gm. CO_2 and 0.276 gm. H_2O.
0.0020 " " " in Van Slyke micro-apparatus 0.59 cc. N at 14°C. and 748 mm.

	Calculated for $C_{18}H_{21}N_5O_{16}P_2Ba_2+2H_2O$:	Found:
C	22.69	22.53
H	2.62	3.19
N	7.63	7.23
P	6.76	6.24
Ba	28.72	28.59
NH_2N	1.53	1.70

The optical rotation of the substance in 2.5 per cent HCl was:

$$[\alpha]_D^{25} = \frac{+0.50° \times 2.5}{1 \times 0.100} = +12.5° \text{ for the barium salt} = +18.52° \text{ for the free dinucleotide.}$$

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THE REMOVAL OF NITRIC ACID FROM SOLUTIONS OF ORGANIC COMPOUNDS.

By P. A. LEVENE and G. M. MEYER.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 26, 1917.)

The lack of a convenient method for removing nitric acid has restricted to a minimum its use in organic and biological chemistry. In this laboratory we have felt the disturbing presence of nitric acid particularly in the preparation of the salts of anhydrotalonic and anhydrogalactonic acids, and of similar substances.

In inorganic analysis several methods for removing nitric acid are used. The majority of them are based on the process of reduction. Unfortunately the reduction is frequently carried out under conditions that would act destructively on the organic material, if any were present. The problem as it presented itself to us consisted in the selection of a method of reduction which could be carried out in a solution with a reaction in the neighborhood of neutrality and at ordinary temperature. Besides it was desired to select such reagents as were readily removable from the solution.

Of the reducing agents generally recommended for this purpose the following were employed: zinc dust, zinc-copper couple,[1] iron and sulfuric acid,[2] and aluminum amalgam.[3] By means of zinc dust or zinc-copper couple in solutions approaching neutrality the reduction proceeded very slowly and never reached completion. On the other hand, by means of aluminum amalgam the reduction is completed in 6 hours. When the process of reduction of nitric acid by means of aluminum amalgam was recommended as a method for the quantitative determination of nitric acid, it was assumed that the acid was reduced completely

[1] Williams, M. W., *J. Chem. Soc.*, 1881, xxxix, 100, 144.

[2] Alberti and Hempel, *Z. angew. Chem.*, 1892, 101.

[3] Ormandy, R., and Cohen, J. B., *J. Chem. Soc.*, 1890, lvii, 811.

to ammonia. Under the conditions here described only about 40 per cent of the nitric acid was reduced to ammonia; the remaining 60 per cent escaped the solution undoubtedly in the form of lower oxides of nitrogen. In this connection it was interesting to note that the rate of disappearance of nitric acid was much higher than that of ammonia formation, thus showing that in the course of the reduction the proportion of the lower oxides compared with that of N_2O_5 was continually increasing. Because of this it was deemed important to show experimentally that when the solution became free from nitric acid it was also free from N_2O_3.

Theoretically one could not expect to find an absence of nitric acid where nitrous acid was present, since, in aqueous solution, nitrites always assume the following equilibrium:

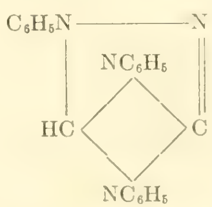
$$3MNO_2 + H_2O = MNO_3 + 2NO + 2MOH.$$

However, to remove all possible doubt, the reduced solution was analyzed for both nitric and nitrous acids.

Nitric acid was estimated by means of "nitron."[4] At the end of the experiment a test for nitric acid was also made by means of brucine. The test was always negative.

The absence of nitrous acid was shown by means of a solution of potassium permanganate. After standing with the reduced solution for 1 hour no reduction of the permanganate solution could be detected. Hence it was proven that aluminum amalgam could be employed conveniently for removing nitric and nitrous acids.

[4] *Diphenyl-endanilo-dehydrotriazol,* with which it forms an insoluble precipitate.



Busch, M., *Ber. chem. Ges.*, 1905, xxxviii, 681. Gutbier, A., *Z. angew. Chem.*, 1905, xviii, 494.

When the process is applied to the removal of nitric acid from organic mixtures it becomes necessary also to remove the introduced reagents or their transformation product. The following procedure was adopted.

1. The acidity of the solution is determined by titration of a small sample and the solution is then neutralized by means of barium hydroxide.

2. About 2 gm. of freshly prepared aluminum amalgam are added for each gm. of nitric acid. The reduction is allowed to proceed for 8 hours or over night. The solution is aerated during the entire time of reduction.

3. The mixture is filtered from the mercury and aluminum. A slight excess of barium hydroxide is then added to the filtrate, and the mixture is concentrated under diminished pressure, to remove ammonia. Generally the process is completed after the evaporation has been repeated twice.

4. The barium is removed quantitatively and the filtrate is ready for further operations.

EXPERIMENTAL.

Determination of Nitric Acid with Nitron.—The solution of barium nitrate containing the equivalent of about 100 mg. of nitric acid was acidified with sulfuric acid and filtered from barium sulfate. To the filtrate 12 to 15 cc. of 10 per cent nitron solution in 5 per cent acetic acid were added and then the mixture was cooled in an ice bath. The precipitate was transferred to a weighed alundum crucible, washed with ice cold water, dried at 105–110°, and weighed. The weight of nitron nitrate $\times$ 0.168 = nitric acid.

Determination of Ammonia.—Ammonia was determined by distilling aliquot portions of the solution, made alkaline, into 0.1 N acid. In those experiments in which the nitrate solutions were aerated during the reduction, the diluted acid solution into which the air was passed was added to the sample taken from the reaction flask, so that the determination in all experiments represents the total quantity of ammonia which was formed during the period indicated.

Aluminum Amalgam.—Sheets of aluminum foil about 4 by 6 inches are passed through a flame to remove the grease and im-

mersed in a shallow bath of about 3 per cent solution of mercuric chloride. In a few minutes the surface of the foil is covered with mercury. The foil is immediately washed in running water and is at once transferred to the nitrate solution.

Reduction of Nitrate Solutions.—Dilute solutions of barium nitrate were placed in flasks with aluminum amalgam. These flasks were fitted with rubber stopper and tubing so that the ammonia which might be generated would be collected in wash bottles containing dilute acid. In some experiments the ammonia was drawn into the dilute acid by a slow current of air.

Experiment 1.—4 gm. of barium nitrate were dissolved in 200 cc. of water and 2 gm. of aluminum amalgam were added and allowed to remain for about 2 hours without aeration. After 12, 36, and 60 hours, samples were withdrawn for analysis. The results are shown in Table I.

TABLE I.

Time.	Theory NH_3 obtainable.	NH_3 found.	Theory.
hrs.	*gm.*	*gm.*	*per cent*
0	0.520		
12		0.0774	14.9
36		0.1449	27.9
60		0.0483	37.6

Experiment 2.—8 gm. of barium nitrate were dissolved in 400 cc. of water and 4 gm. of aluminum amalgam were added. This solution was aerated and the ammonia was determined after 12, 36, and 60 hours. The results are given in Table II.

TABLE II.

Time.	Theory NH_3 obtainable.	NH_3 found.	Theory.
hrs.	*gm.*	*gm.*	*per cent*
0	1.0400		
12		0.2734	27.0
36		0.1049	31.63
60		0.0219	38.00

Experiment 3.—2 gm. of barium nitrate were dissolved in 100 cc. of water, 1 gm. of aluminum amalgam was added, and aerated

for 14 hours, after which time the nitric acid test with nitron was negative as well as with brucine.

Experiment 4.—8 gm. of barium nitrate were dissolved in 400 cc. of water and 6 gm. of aluminum amalgam were added. After 4 hours the nitric acid test with the nitron and brucine was negative.

Experiment 5.—50 cc. of 2.3 per cent nitric acid and 1 gm. of aluminum amalgam were allowed to stand for 16 hours. After 4 hours, 15.9 per cent acid was not reduced and after 16 hours 4.6 per cent nitric acid remained.

Experiment 6.—10 gm. of barium nitrate were dissolved in 500 cc. of water and 7 gm. of aluminum amalgam were added. Then additional amalgam was added every 2 hours as indicated in the table, and at each 2 hour interval a sample of the liquid was withdrawn for the determination of nitric acid and ammonia. As Table III shows, no nitric acid remained after 6 hours although only 40 per cent of the nitric acid was obtained in the form of ammonia.

TABLE III.

	Nitric acid determined with nitron.						Ammonia (NH_3).				
Time.	Aluminum amalgam.	Present.	Reduced.	Present.	Reduced.	Calculated from HNO_3 present.	NH_3 determined.	Total produced.	NH_3 obtained during each interval.	NH_3 obtained from beginning.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
0	7.0	5.660	0.000	100.0	000.0	1.527				
2	3.0	1.392	4.268	24.0	76.0		0.1205	0.1205	7.90	7.60
3	2.0	0.558	5.102	9.8	90.2		0.1475	0.2680	9.68	17.60
4	0.5	0.0045	5.655	0.79	99.21		0.1665	0.4345	10.90	28.50
5	0.5	Trace.	Nearly all.	Trace.	99.99		0.1904	0.6249	12.48	40.96
6	0.5	None.	5.660	None.	100.00					

Experiment 7.—10 gm. of barium nitrate were dissolved in 500 cc. of water and 7 gm. of aluminum amalgam were added. The solution was aerated and allowed to stand for 14 hours. The mixture was filtered and the filtrate tested for nitric and nitrous acids. Nitric acid was found absent both with brucine and nitron. 20 cc. of 0.1 N potassium permanganate acidified with

sulfuric acid were added to 20 cc. of the filtrate and the unused permanganate was titrated, according to the method of Volhard, with potassium iodide. No nitrous acid was found.

THE PREPARATION OF LYXOSE.

By E. P. CLARK.

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Of the methods for preparation of lyxose the one introduced by Ruff and Ollendorff[1] is the most convenient. This consists in the oxidation of calcium *d*-galactonate by means of hydrogen peroxide, using ferric acetate as a catalyst. The details of the directions as given by Ruff and Ollendorff are not sufficient for the preparation of this pentose on a large scale. In the course of the past year over 3,000 gm. of the sugar were prepared in this laboratory, and the process has been gradually improved so that finally a yield of 195 gm. of pure crystalline lyxose was obtained from 1,500 gm. of calcium galactonate. The conditions as finally adopted are reported here in the hope that they may prove useful to other workers.

EXPERIMENTAL.

500 gm. of calcium galactonate were dissolved in 2 liters of boiling water, and 3 liters of 3 per cent hydrogen peroxide added. The solution was cooled to about 35° and 75 cc. of ferric acetate solution[2] were added, which soon caused a vigorous reaction. After the reaction was completed, which was indicated by the solution acquiring a deep purple color, it was allowed to cool. The solution was filtered and evaporated in vacuum to about 1,200 cc. To the concentrated solution 4 liters of 95 per cent alcohol were added with constant stirring. This precipitates a gummy mass which is hard to handle but if a current of air is blown through the suspension for a short time, all the gummy particles settle out, leaving a clear solution. This solution was

[1] Ruff, O., and Ollendorff, G., *Ber. chem. Ges.*, 1900, xxxiii, 1798.

[2] National Formulary, 3rd edition, Baltimore, 1906, p. 219.

filtered with suction. The gum remaining in the jar, which was drained as dry as possible, and the precipitate on the filter, were then dissolved in about 900 cc. of hot water. This may be readily accomplished in 4 or 5 minutes by heating the mixture, with constant stirring, to about 60–65° by means of live steam. After the gum had dissolved, the liquid was cooled to room temperature and precipitated with 4 liters of 95 per cent alcohol, as above.

Three lots of 500 gm. each were treated in this way and the combined residues from them were reoxidized in the following manner: They were dissolved in several liters of hot water, allowed to cool, and filtered. The filtrate was evaporated to dryness *in vacuo* to remove all the alcohol. The residue was then dissolved in about 2 liters of hot water by means of live steam and 5 liters of hydrogen peroxide were added. The solution was cooled to 35° and 80 cc. of ferric acetate solution were added. After the reaction was complete the solution was filtered and concentrated, then precipitated with 95 per cent alcohol as previously described. The residues were dissolved in 1 liter of water and again precipitated with 4 liters of 95 per cent alcohol.

The combined alcoholic extracts resulting from the above procedure were then evaporated *in vacuo* to 1 liter. 95 per cent alcohol was added with constant stirring until a permanent precipitate was formed; about 1.5 liters of alcohol were required. This solution was poured into 9 liters of absolute alcohol with constant agitation. The precipitate formed was filtered off, drained as dry as possible, and the filtrate evaporated *in vacuo* to a thick syrup (about 700 cc.). This syrup was taken up in 8 liters of absolute alcohol and 3.5 liters of dry ether *slowly* added with constant stirring, which precipitates a further quantity of calcium salts and other reaction products. The filtered solution was evaporated *in vacuo* to 500 cc., seeded with a few crystals of lyxose, and allowed to crystallize in a desiccator. Often the syrup can be made to crystallize spontaneously without seeding by scratching the inside of the beaker.

The crystals were filtered with suction and washed first with absolute alcohol and then with dry ether. The yield was generally 150 to 165 gm. of pure dry sugar. Lyxose may be readily

recrystallized with little loss from four to five parts of boiling absolute alcohol. No attempt was made to work over the mother liquors from the crystallization of lyxose, as it was found that they could be used directly for the preparation of lyxosimine. The syrup gave a yield of lyxosimine corresponding to about 55 or 60 gm. of lyxose. Yields of pure crystalline lyxose from lots of 1,500 gm. of calcium galactonate have been obtained as high as 195 gm., but in these cases a corresponding diminution of lyxosimine has been obtained from the mother liquors, so that almost invariably the total yield corresponded to about 210 gm. of lyxose from 1,500 gm. of calcium galactonate.

CHONDROSAMINE AND ITS SYNTHESIS.

By P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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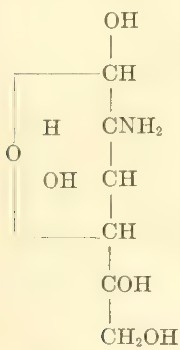
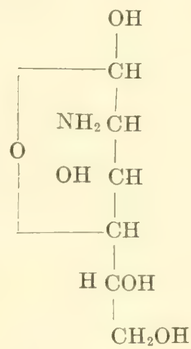
In previous publications[1] the conclusion was reached that chondrosamine had the structure of one of the two lyxohexosamines:



or



The relationship of chondrosamine to galactose was originally based on the following facts:

Chondrosamine formed with phenylhydrazine an osazone indistinguishable from galactosazone. Chondrosamine, on oxidation with bromine or with mercuric oxide, formed chondrosaminic acid. This acid on oxidation with nitric acid (subsequent to deamination) yielded anhydromucic acid. On the other hand direct oxidation of chondrosamine (subsequent to deamination) led to an optically active dicarboxylic acid which was assumed to be anhydrotalomucic acid. Furthermore, chondrosamine on oxidation with bromine or with mercuric oxide (subsequent to deamination) gave rise to anhydrotalonic acid. Chondrosaminic acid under the same treatment formed the isomeric anhydro-

[1] Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1913, xv, 159; 1914, xviii, 127, 240; 1915, xx, 434.

galactonic acid. In a measure the proof seemed sufficient to establish the configuration of the new aminohexose. Yet a scrutiny of the evidence makes it clear that only one point of evidence was absolutely beyond dispute; that is, the identity of the chondrosamine osazone with galactosazone. True, the two anhydrotetrahydroxyadipic acids, one obtained from chondrosamine and the other from lyxohexosaminic acids, seemed identical. However, this conclusion was based on their melting points being identical and on the absence of optical activity in one and the other. These points may be considered ample proof, but additional evidence was desirable. Again the anhydrotalonic and anhydrogalactonic acids had been prepared only in form of their brucine salts, and although the differences in their optical rotation were in full agreement with the assumed structure of the two salts, yet more direct evidence in support of the assumption was much wanted.

The most convincing proof possible would be of course the synthesis of all the substances derived from chondrosamine, using lyxose as the starting material. The synthesis of the sugar has now been accomplished, and with it for every known derivative of the natural sugar a corresponding derivative of the synthetic substance has been obtained. Thus the problem of the configuration of chondrosamine is definitely solved.

Chondrosamine Hydrochloride.—This was prepared last year by the reduction of lyxohexosaminic acid. Its optical rotation was then found to be $[\alpha]_D^\circ = +62.69$ to $+91.10°$. The rotation of chondrosamine hydrochloride was determined by Levene and La Forge, $[\alpha]_D^\circ = +129.50$ to $93.82°$. This difference in rotation and the fact that chondrosaminic acid differed in its rotation from the synthetic lyxohexosaminic acid led originally to the conclusion that the natural and the synthetic sugars were epimers. However, the fact that the rotation of the two sugars reached the same value at the state of equilibrium suggested the possibility that they were α and β forms of the same sugar. Hence the rotation of the recently prepared chondrosamine hydrochloride was redetermined, and was found identical with that of the synthetic product in the proximity of $[\alpha]_D^\circ = +57$ to $93°$. Many samples crystallized under different conditions were tested, always with the same result. This was a surprising and puzzling

finding; and although there was no doubt in our mind as to the correctness of the early measurement, since all readings in this laboratory are taken by two observers, yet to eliminate all possible doubt the rotation of the original material was again measured and was found as originally recorded $[\alpha]_D^0 = + 129.0$ to $95.0°$. It is interesting to note that in the early work of Levene and La Forge the first form occurred on three occasions whereas in subsequent work it could not be obtained again. It is also worthy of note that the value of the molecular rotation of the end carbon atom of the two amino sugars calculated according to Hudson's formula is in good agreement with the value calculated by Hudson for the end carbon atom of other hexoses.

Chondrosaminic Acid.—The observations on chondrosaminic acid and on the acid obtained synthetically originally seemed puzzling and confusing. By the action of hydrocyanic acid on arabinosimine, Fischer and Leuchs[2] obtained pure glucosaminic acid. Because of this it was thought that lyxohexosaminic acid obtained by the action of hydrocyanic acid on lyxosimine was also a uniform substance. The synthetic acid originally obtained in this manner had $[\alpha]_D^0 = - 3.58$ to $- 20.7°$. which differed from chondrosaminic acid with $[\alpha]_D^0 = - 16.15$ to $- 29.2°$. On the other hand, the reduction of the synthetic acid led to the same chondrosamine, which on oxidation gave rise to chondrosaminic acid. The confusion, however, was cleared up when it was found that the synthetic sugar on oxidation formed the same amino acid as the natural sugar.

Parallel measurements of the optical rotation were made on samples of the acids from the natural and synthetic sugars and were found identical, $[\alpha]_D^0 = - 17.94$ to $- 31.89°$. In the light of this, lyxohexosaminic acid has to be regarded as a mixture of the epimeric acids. Indeed, the material prepared this year had $[\alpha]_D^0 = - 1.85$ to $- 8.78°$., which differed from the $[\alpha]_D$ of the original synthetic acid. Obviously under varying conditions of experiment different proportions of the epimeric acids are formed.

α, α_1-Anhydromucic Acid, and α, α_1-Anhydrotalomucic Acids.—Inactive anhydrotetrahydroxyadipic acids were obtained from chondrosamine and from the synthetic lyxohexosamnic acids.

[2] Fischer, E., and Leuchs, H., *Ber. chem. Ges.*, 1903, xxxvi, 24.

So long as the latter was considered an individual substance different from chondrosaminic acid, the observation needed special interpretation. Since, however, it was demonstrated that the synthetic acid was a mixture of two epimers, one being chondrosaminic acid, the puzzling element of the observation was eliminated, the identity of the two anhydrotetrahydroxyadipic acids became evident, and their structure could be no other than that of anhydromucic acid.

The structure of the optically active tetrahydroxyadipic acids is manifested from the fact that the natural sugar gives rise to the acid with exactly the same properties as that derived from the synthetic sugar. Since the structure of the latter is obvious from its origin the structure of the former as anhydrotalomucic becomes certain.

α,α_1-Anhydrotalonic and α,α_1-Anhydrogalactonic Acids.—Since the structure of the α, α_1-anhydrotalomucic and α, α_1-anhydromucic acid has been established it became an easy task to prove the structure of the two monocarboxylic acids. The anhydrotalonic should be convertible into anhydrotalomucic acid, and anhydrogalactonic into anhydromucic acid. The brucine salts of two acids were previously obtained, one with a melting point of 218° and $[\alpha]_D^{20} = -12.4$, and the other with a melting point of 244° and $[\alpha]_D^{20} = -9.40$. It was then shown that the former on oxidation gives anhydrotalomucic while the latter forms anhydromucic acid. The former was assumed to possess the structure of anhydrotalonic, the latter of anhydrogalactonic acid. The assumption was fully justified and stands correct in the light of the new evidence.

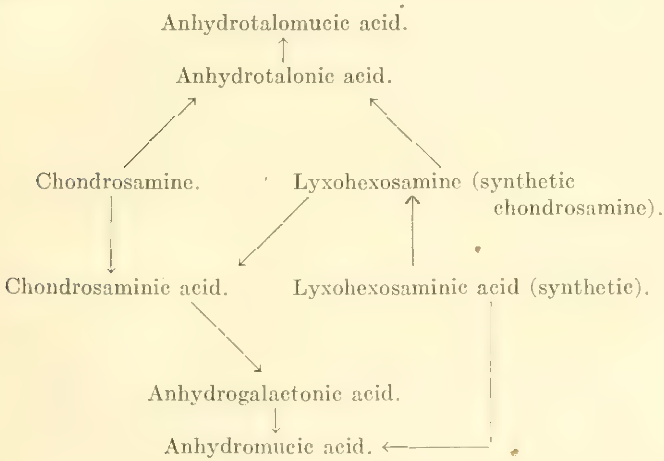
It may be mentioned in this place that formerly the formation of anhydrotalonic acid from the synthetic lyxohexosaminic had seemed puzzling. In view of the fact that lyxohexosaminic acid is recognized as a mixture of two epimers, the source of the anhydrotalonic is to be looked for in one of the two epimers, namely, in epichondrosaminic acid.

Pentacetyl Derivatives.

As a final point proving the identity of the two sugars may be mentioned the fact that they yield the identical pentacetates. Both the α and the β forms obtained by Hudson and Dale from the

natural product were then obtained from the synthetic sugar. It may be noted in this place that Hudson and Dale designated the more soluble pentacetate of chondrosamine with $[\alpha]_D^{20} = 101.3°$ as the β form and the more insoluble with $[\alpha]_D^{20} = 11.0°$ as the α form. This nomenclature was perfectly justified when it was assumed that chondrosamine was *l*-ribohexosamine. Inasmuch as chondrosamine is now recognized as *d*-lyxohexosamine, the form with the higher rotation has to be named the α form, $[\alpha]_D^{20} = 101.3°$, and the one with $[\alpha]_D^{20} = +11.0$, the β form.

The mutual relationship of the derivatives of chondrosamine on one hand and of the derivatives and of the parent substances of the synthetic sugar on the other is represented in the following diagram.



EXPERIMENTAL.

Preparation of Synthetic Chondrosamine Hydrochloride.—The process was essentially the same as previously described.[3] A slight improvement was introduced in that all solutions were concentrated at a temperature of the water bath not exceeding 40°C. It was found convenient to recrystallize the sugar by dissolving it in a minimum amount of water and adding ethyl alcohol saturated with hydrochloric acid. About 35.0 gm. of the synthetic

[3] Levene, P. A., *J. Biol. Chem.*, 1916, xxvi, 143, 155.

sugars were prepared. The specific rotation of the substance was the following.

Initial. Equilibrium.

$$[\alpha]_D^0 = \frac{+1.50 \times 2.0392}{1 \times 0.0516} = +59.30° \quad [\alpha]_D^{25} = \frac{+2.50 \times 2.0392}{1 \times 0.0516} = +98.80°$$

α and β Forms of Chondrosamine Hydrochloride.—A sample of chondrosamine recrystallized out of water and ethyl alcohol saturated with hydrochloric acid.

Initial. Equilibrium.

$$[\alpha]_D^0 = \frac{+1.54 \times 2.0385}{1 \times 0.0516} = +53.14 \quad [\alpha]_D^{25} = \frac{+2.28 \times 2.0392}{1 \times 0.0514} = +90.42$$

A sample was dissolved in a minimum amount of water, glacial acetic acid was added until the substance began to crystallize, the mixture brought to a boil, and filtered. A solution was made of exactly 2.5 cc. volume.

Initial. Equilibrium.

$$[\alpha]_D^0 = \frac{+3.04 \times 2.5}{1 \times 0.125} = +60.4° \quad [\alpha]_D^{25} = \frac{4.5 \times 2.5}{1 \times 0.125} = +90.0°$$

A sample crystallized out of a minimum amount of aqueous hydrochloric acid.

Initial. Equilibrium.

$$[\alpha]_D^0 = \frac{+2.60 \times 2.5}{1 \times 0.1272} = +57.10° \quad [\alpha]_D^{25} = \frac{+4.80 \times 2.5}{1 \times 0.1272} = +94.20°$$

No attempt was made to explain the slight discrepancies in the solutions since the principal object was to determine conditions controlling the formation of either one of the forms. As all attempts to obtain a sample with the original rotation failed, the rotation of the original material was redetermined. Dr. J. López-Suárez and Dr. G. M. Meyer controlled the reading.

Initial. Equilibrium.

$$[\alpha]_D^0 = \frac{+2.50 \times 2.5}{1 \times 0.0500} = +129.0° \quad [\alpha]_D^{25} = \frac{+1.90 \times 2.5}{1 \times 0.500} = +96.0°$$

Calculating on the basis of Hudson's formula, the molecular rotation of the end carbon atom $= \frac{(129.0 - 51.0)}{2} 215.5 = 8,400.$ The value found by Hudson for hexoses was in the neighborhood

of 8,000. The original form is to be regarded as the α and the new as the β form.

Chondrosaminic Acid from Natural and Synthetic Sugars.—This acid was obtained from both the natural and synthetic sugar by oxidation with mercuric oxide. The conditions given by Pringsheim and Ruschmann[4] for oxidation of glucosamine had to be modified.

4.0 gm. of the synthetic sugar were dissolved in 62.0 cc. of water, to the solution 20.0 gm. of mercuric oxide were added, and the mixture was warmed on the water bath for 6 minutes. The reaction product was filtered immediately, the filtrate was freed from mercury by means of hydrogen sulfide, the filtrate from the sulfide concentrated to a small volume, under diminished pressure, when the acid crystallized in the distilling flask. The substance was recrystallized once. It did not melt, but turned light brown at 190°, the same as the acid obtained from the natural sugar. A parallel measurement of the rotation of each product gave the following results.

Natural product.

Initial in 2.5 per cent HCl. Equilibrium.

$$[\alpha]_D^0 = \frac{-0.90 \times 2.5}{1 \times 0.1254} = -17.94^\circ \qquad [\alpha]_D^{25} = \frac{-1.60 \times 2.5}{1 \times 0.1254} = -31.89^\circ$$

Synthetic.

Initial in 2.5 per cent HCl. Equilibrium.

$$[\alpha]_D^0 = \frac{-0.94 \times 2.5}{1 \times 0.1256} = -17.94^\circ \qquad [\alpha]_D^{25} = \frac{-1.60 \times 2.5}{1 \times 0.1256} = -31.87^\circ$$

. Lyxohexosaminic acid.

$$[\alpha]_D^0 = \frac{+0.37 \times 15.0}{2.0 \times 1.496} = +1.85^\circ \qquad [\alpha]_D^{25} = \frac{-1.73 \times 15.0}{2.0 \times 1.4946} = -8.75^\circ$$

The analysis of the synthetic acid gave the following results.

0.1050 gm. of substance gave 0.1426 gm. CO_2 and 0.0618 gm. H_2O.

	Calculated for $C_6H_{13}NO_3$:	Found:
C	36.92	37.03
H	6.66	6.58

Thus the identity of the two chondrosaminic acids is establisbed.

[4] Pringsheim, H., and Ruschmann, G., *Ber. chem. Ges.*, 1915, xlviii, 680.

Anhydrotalonic Acid.—The acid was previously obtained in small quantities as a brucine salt. The brucine salt was prepared in larger quantities from the natural sugar and from the sugar obtained from lyxohexosaminic acid. Lots of 30.0 gm. of chondrosamine hydrochloride were dissolved in 150.0 cc. of water. To the solution 30.0 gm. of silver nitrite and a few drops of hydrochloric acid were added. The mixture was allowed to react for 6 hours. The silver chloride was then removed by filtration, the filtrate was placed on a boiling water bath for 5 minutes, then treated with a slight excess of hydrochloric acid, and again filtered. To the filtrate 65.0 gm. of bromine were added and the mixture was allowed to stand at room temperature for 3 days. The remaining traces of bromine were removed by shaking the solution with mercury, and the hydrobromic acid by means of lead carbonate and silver carbonate. Finally the nitric acid still present in the solution was removed by the aluminum amalgam method. The resulting solution was transformed into the brucine salt in the usual manner. The solution of the brucine salt was concentrated to a small volume when, on cooling, the brucine salt was crystallized out. When first obtained the substance was readily soluble in methyl alcohol. However, on repeated recrystallization its solubility diminished, so that towards the end it dissolved only in a large volume of boiling methyl alcohol. The salt then crystallized in large polygonal prisms. The melting point was 218°C. and the optical rotation was the following.

$$\left[\alpha\right]_{D}^{20} = \frac{-0.63^\circ \times 10.0}{2 \times 2.522} = -12.4^\circ$$

The analysis of the substance gave the following results.

0.1063 gm. of substance on drying under diminished pressure at the temperature of xylene vapors lost 0.003 gm. of water.

	Calculated for $C_{29}H_{33}N_2O_{10}+H_2O$:	Found:
H_2O	3.11	3.58

0.1025 gm. of the dry substance gave 0.2284 gm. CO_2 and 0.0569 gm. H_2O.

	Calculated for $C_{29}H_{36}N_2O_{10}$:	Found:
C	60.80	60.78
H	6.10	6.15

From the synthetic lyxohexosaminic acid the substance was prepared in the following manner. 30 gm. of the acid were dissolved in a solution of 200.00 cc. of water and 40.0 cc. of 10 per cent hydrochloric acid. 40.0 gm. of silver nitrite were added. The following morning 10.0 gm. of silver nitrite and 10.0 cc. of 10 per cent hydrochloric acid were added. After 30 hours from the beginning of the experiment, the silver chloride was removed by filtration and other remaining silver by hydrogen sulfide. From the filtrate the nitric and nitrous acids were removed by the aluminum amalgam method.

It had the following composition.

0.1012 gm. of substance on drying in a xylene bath under diminished pressure lost 0.0038 gm. of H_2O.

0.0974 gm. of substance, dried, on combustion gave 0.2158 gm. CO_2 and 0.0562 gm. H_2O.

	Calculated for $C_{29}H_{36}N_2O_{10}H_2O$:	Found:
H_2O	3.11	3.75

	Calculated for $C_{29}H_{36}N_2O_{10}$:	Found:
C	60.80	60.42
H	6.10	6.45

The substance had a melting point of 218°C. and the following rotation.

$$[\alpha]_D^{20} = \frac{-0.655^\circ \times 10}{2 \times 2.66} = -12.3$$

Thus the identity of the acid obtained from natural chondrosamine and of the one obtained synthetically was established. In order to prove its identity with α, δ-anhydrotalonic acid it was oxidized by means of nitric acid. 21.0 gm. of the brucine salt were freed from brucine by means of barium hydroxide and chloroform. After the removal of barium, the solution was concentrated to 40.0 cc., an equal volume of concentrated nitric acid was added, and the solution was boiled over a free flame until a lively evolution of red fumes set in. The solution was then transferred to a large clock glass and evaporated to dryness. The residue was dissolved in water and again evaporated to dryness. This operation was repeated. The reaction product was converted into the calcium salt. The salt was recrystal-

lized twice. Each time the calcium salt was decomposed by a little less than the calculated amount of oxalic acid, and then reconverted into the calcium salt.

0.1008 gm. of the air-dry substance on drying in a xylene bath under diminished pressure lost 0.0130 gm. of H_2O.

	Calculated for $C_6H_8O_8C_6+3H_2O$:	Found:
H_2O	12.68	12.90

0.0878 gm. of dry substance gave on combustion 0.0936 gm. CO_2 and 0.0274 gm. H_2O.

0.0896 gm. of the substance gave 0.0206 gm. CaO.

	Calculated for $C_6H_8O_7Ca+H_2O$:	Found:
C	29.03	29.07
H	3.22	3.49
Ca	22.58	22.99

The optical rotation of the substance in a 10 per cent solution of HCl was the following.

$$[\alpha]_D^{20} = \frac{-0.30° \times 2.50}{1 \times 0.100} = -7.5°$$

Thus the anhydrohexonic acid obtained from lyxohexosaminic acid yields on further oxidation an optically active anhydrotetrahydroxyadipic acid; hence it possesses the structure of anhydrotalonic acid.

Anhydrogalactonic Acid.—10 gm. lots of chondrosaminic acid were treated in the same manner as lyxohexosaminic acid in the above experiment. The brucine salt obtained in this manner melted at 244°, and had the following optical rotation.

$$[\alpha]_D^{20} = \frac{-0.47° \times 10.0}{2 \times 2.508} = -9.37°$$

The composition of the substance was the following.

0.0988 gm. of the substance, dried in a xylene bath, gave 0.2196 gm. CO_2 and 0.0576 gm. H_2O.

	Calculated for $C_{29}H_{26}N_2O_{10}$:	Found:
C	60.80	60.73
H	6.10	6.53

10.0 gm. of the brucine salt were freed from brucine as in the above experiment. The brucine-free solution was evaporated

to 25 cc. and then diluted with an equal volume of concentrated nitric acid and boiled over free flame until the volume was reduced to about 20.0 cc., then 10.0 cc. of nitric acid were again added, and the solution was again boiled, over free flame. When the solution was concentrated to 20.0 cc. it was transferred to a clock glass and concentrated on a boiling water bath to dryness. The substance immediately crystallized. It was redissolved in water and again evaporated. The operation was repeated once more. The final crystalline residue was dissolved in hot acetone and very little ether was added, when a small amorphous precipitate formed. This was removed by filtration, and the filtrate was allowed to crystallize. The crystals were filtered, redissolved in hot acetone, and allowed to crystallize. The final product melted at 205°C.

0.1600 gm. of the substance dissolved in 2.5 cc. showed no optical activity.

On the basis of this the identity with anhydrogalactonic acid of the monohydroxy acid obtained from chondrosaminic acid is established.

α,α_1-*Anhydrotalomucic Acid from the Synthetic Sugar.*—60 gm. 6.0
of the synthetic lyxohexosamine hydrochloride were dissolved in 30.0 cc. of water containing 1 cc. of hydrochloric acid. 8.0 gm. of silver nitrate were added and the mixture was allowed to stand for 6 hours. It was then filtered. The silver was removed from the filtrate by means of hydrogen sulfide. The clear filtrate from the silver sulfide was concentrated to 20.0 cc., cooled to 0°C., and diluted with 20.0 cc. of nitric acid also cooled at 0°C. The solution was allowed to stand over night. It was then boiled over a free flame until a lively evolution of yellow fumes set in. The solution was then transferred to a clock glass, evaporated on a water bath, and the calcium salt was then prepared in the usual way. The salt was reprecipitated twice and had the following composition.

0.1054 gm. of substance on drying in a xylene bath lost 0.0130 gm. H_2O.

	Calculated for $C_6H_8O_6Ca+2H_2O$:	Found:
H_2O	12.68	12.33

0.0924 gm. of the dry substance gave on combustion 0.0982 gm. CO_2 and 0.0298 gm. H_2O and 0.0924 gm. CaO.

	Calculated for $C_6H_6O_7Ca+H_2O$:	Found:
C	29.03	28.98
H	3.22	3.60
Ca	22.58	23.05

The optical rotation of the substance is the following.

Initial.

$$[\alpha]_D^{20} = \frac{-0.32° \times 2.5}{1 \times 0.100} = -8.0°$$

Equilibrium.

$$[\alpha]_D^{20} = \frac{-0.32° \times 2.5}{1 \times 0.100} = -8.0°$$

in 10 per cent HCl.

Hence the substance is α,α_1-anhydrotalomucic acid. It is identical with the substance previously obtained from natural chondrosamine.

Pentacetyl Derivative of the Synthetic Chondrosamine.—The substance was prepared from the natural chondrosamine by Hudson and Dale.[5] Practically the same conditions were followed in this work.

In 30.0 cc. of acetic anhydride 5.0 gm. of zinc chloride were dissolved. To this solution 5.0 gm. of the hydrochloride were added and the mixture was warmed gently until a lively reaction developed. The reaction was kept up for 2 minutes, then the reaction product was poured into 100 cc. of water cooled to 0°C. The mixture was neutralized with potassium bicarbonate, transferred to a separatory funnel, and extracted with chloroform. The chloroform extract was washed with water. Over the chloroform a layer of crystals appeared which were insoluble in water. The crystals consisted of the more insoluble fraction of the pentacetates. The chloroform extract was evaporated to dryness *in vacuo*, the residue was recrystallized out of alcohol, and from the mother liquor a second crop of crystals was obtained. The top fraction consisted of the pure α form while the most soluble form was practically the pure β form.

The α form turned slightly brown at 232° and melted with decomposition at 237°C. (corrected). Its optical rotation in chloroform solution was the following.

[5] Hudson, C. S., and Dale, J. K., *J. Am. Chem. Soc.*, 1916, xxxviii, 1431.

$$[\alpha]_D^{20} = \frac{+ 0.07 \times 20.0}{2 \times 0.0802} = + 8.75°$$

A similar fraction from natural chondrosamine had a melting point of 235°C. and the following optical rotation.

$$[\alpha]_D^{20} = \frac{0.09 \times 20.0}{2 \times 0.075} = + 12.0°$$

Hudson and Dale found for their β form a melting point of 235° (with decomposition) $[\alpha]_D = + 11.00°$.

The β form was apparently not quite pure, but taking into consideration the small quantity of starting material it is rather surprising that each form could be separated with so little difficulty.

The melting point of the β form was very sharp at 197°C. and the optical rotation in chloroform solution was

$$[\alpha]_D^{20} = \frac{+ 0.90 \times 20.0}{2 \times 1.000} = + 90.0°$$

Hudson and Dale found for the α form the melting point 182–183°C. and $[\alpha]_D$ 101.3°.

The composition of the pentacetyl derivative was the following.

0.1014 gm. of the substance gave 0.1834 gm. CO_2 and 0.0560 gm. H_2O.

	Calculated for $C_6H_8NO_5(CH_3CO)_5$:	Found:
C	49.49	49.32
H	5.96	6.18

THE RELATION BETWEEN THE CONFIGURATION AND ROTATION OF EPIMERIC MONOCARBOXYLIC SUGAR ACIDS.

III. THE PHENYLHYDRAZIDES.

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In previous publications[1] it was demonstrated that the direction of the optical rotation of the α carbon atom of a pair of epimeric monocarboxylic sugar acids is determined by its configuration. When the hydroxyl of the α carbon atom is in the same position as in *d*-gluconic acid (on the right) the direction of the rotation is right, while when it is in the same position as in *d*-mannonic acid the direction of the rotation is left. The rule was found valid for phenylhydrazides, brucine, strychnine, and sodium and calcium salts.

In the early phase of the work it seemed that in salts of different acids with the same base and possibly in all derivatives of the sugar acids, the value of the molecular rotation of the α carbon atom was constant. If that were true the magnitude of the rotation of the α carbon atom of a given salt would serve as an index of its purity. Following our observation, Hudson[2] succeeded in demonstrating that in phenylhydrazides the magnitude of the rotation of the β, γ, and δ carbon atoms of several monocarboxylic acids were insignificant as compared with that of the α carbon atom, so that the direction of the rotation of the phenylhydrazide could be determined by that of the α carbon atom. Because of this Hudson suggested that, *vice versa*, the configuration of the α carbon atom in a given sugar acid may be

[1] Levene, P. A., *J. Biol. Chem.*, 1915, xxiii, 145. Levene, P. A., and Meyer, G. M., *ibid.*, 1916, xxvi, 355.

[2] Hudson, C. S., *J. Am. Chem. Soc.*, 1917, xxxix, 462.

determined by the direction of the rotation of its phenylhydrazide. Wherever the phenylhydrazide is obtainable this undoubtedly is correct. In our first note on this subject only one pair of epimeric phenylhydrazides was discussed, and further work was in progress when Hudson's article appeared. We did not discontinue our work for the reason that it has furnished some interesting results regarding the numerical value of the α carbon atom in different derivatives of the same acid.

As already mentioned, it was expected that the molecular rotation of the α carbon atom would be constant in all compounds of such acids, the purity of which could not be questioned. A scrutiny of the empirical values for the molecular rotation of the α carbon atom for the various pairs of epimeric salts revealed considerable variations, and it remained uncertain whether these were occasioned by the impurity of some of the acids.

The maximum rotation of the α carbon atom of a pair of acids is obtained when both acids are perfectly free from the epimeric acid, since the rotation of the α carbon atom of each of two epimers is in opposite directions. There can scarcely be any doubt that derivatives of gluconic and mannonic acids can be obtained in perfect purity, since both acids form crystalline lactones. Hence it was surprising to find that the rotation of the α carbon atom in the pair of allonic and altronic hydrazides was of a higher value than that of the gluconic-mannonic pair. On the other hand, in the gulonic-idonic pair the magnitude of rotation was equal to that of the gluconic-mannonic pair. Hence it is justifiable to accept the purity of idonic acid. On the other hand, the value of the rotation of the α carbon atom in the talonic-galactonic pair was much lower than that of any other pair. Besides, the rotation of both hydrazides was found to be in the same direction. Since galactonic lactone is obtained in a beautifully crystalline form, there exists no doubt as to its purity. On the contrary, the hydrazide of talonic acid was obtained from the brucine salt, and there always existed among workers a certain scepticism as to the purity of this compound. The brucine salt employed in this work has the rotation of $[\alpha]_D^{20} = -26.15°$. This is the highest value recorded for the rotation of the brucine salt of talonic acid.

The scrutiny of the data obtained in the course of this work indicates that the magnitude of the rotation of the α carbon atom is not altogether constant even for the series of phenylhydrazides, but the rule of the relation of direction of the rotation to the configuration of the α carbon atom remains valid.

In the present work all readings were made on two solutions prepared from the same sample so as to reduce to a minimum the experimental error.

Phenylhydrazide.	Author.	C	T°.	[α]	$\frac{A+B}{2}$
Gluconic.		1	85	+18.0	
		1	85	+18.2	
	Nef.[3]	2	20	+12.0	
Mannonic.		1	85	−10.5	14.25°
		1	90	−10.7	
	Hudson.[2]	2	80	− 8.1	
Gulonic.		1	20	+13.45	
		1	20	+13.87	
	Nef.	4	20	+13.74	
Idonic.		1	20	−15.1	14.25°
		1	20	−15.3	
	Nef.	2	20	−12.42	
Galactonic.		1	25	+12.2	
	Nef.	2	20	+10.44	8.25°
Talonic.		0.5	25	+ 4.35	
Allonic.		1	20	+25.88	
		1	20	+25.50	
Altronic.		1	20	−15.8	20.8°
		1	20	−15.9	
Arabonic.		2.5	63	−16.09	
	Hudson.	1	20	−14.5	16.09°
Ribonic.		2.5	63	+16.09	

[3] Nef, J. U., *Ann. Chem.*, 1914, cdiii, 204.

EXPERIMENTAL.

The phenylhydrazides of gluconic, mannonic, galactonic, gilonic, and arabonic acids were prepared in the usual way and were recrystallized out of water. They were obtained in the form of perfectly colorless bright plates.

The idonic, gulonic, talonic, altronic, and ribonic acids were precipitated out of alcoholic solutions by ether. A slightly gelatinous perfectly white substance settled out, which crystallized out of alcohol in the form of colorless plates. Idonic acid was prepared by the method of Van Ekenstein and Blanksma.[4]

With a few exceptions all readings were made in a 2 dm. tube. Practically all readings were made with an accuracy of $\pm 0.00°$.

[4] Van Ekenstein, W. A., and Blanksma, J. J., *Rec. trav. chim. Pays-Bas*, 1908, xxvii, 1.

CEREBROSIDES.

III. CONDITIONS FOR HYDROLYSIS OF CEREBROSIDES.

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The conditions of hydrolysis of cerebrosides followed by most investigators remain as they were introduced by Thudichum,[1] the pioneer worker in this field. Thudichum made use of two methods. One consisted in heating the cerebrosides in a 2 per cent aqueous sulfuric acid solution in sealed tubes for a period varying from 310 to 370 hours. In the second method, the substance was decomposed by heating a solution in ethyl alcohol containing sulfuric acid, under a reflux. These methods remain quite serviceable for experiments of a qualitative nature. However, the methods proved imperfect for an investigation into the quantitative relationships of the components of cerebrosides. Indeed, Thudichum never intended them for that purpose. On the other hand subsequent workers who attempted the quantitative analysis of cerebrosides followed Thudichum's directions without having ascertained the degree of their accuracy.

Early in our work on the hydrolysis of cerebrosides we became aware of the fact that the original methods of hydrolysis demanded improvement from the standpoint of convenience and reliability. The original aqueous hydrolysis was carried out for a period of over 300 hours. It is evident that this prolonged heating must bring about a partial decomposition of the galactose. Again, the alcoholic hydrolysis (partial alcoholysis) was found both inconvenient and inaccurate. Thudichum had demonstrated the presence of some intermediate substances among the products of hydrolysis. Furthermore, the mineral acid in the

[1] Thudichum, J. L. W., Physiological Chemistry of the Brain, London, 1884.

presence of alcohol catalyzes not only the process of splitting the original substances, but also the synthetic processes between alcohol and the cleavage products. Hence the products of reaction on boiling the cerebrosides with an alcoholic solution of a mineral acid are galactose, ethyl galactoside, fatty acids and their ethyl esters, sphingosine, and mono- and diethyl sphingosine. The separation of these substances is not a simple matter.

Because of this, an attempt was made to find conditions of hydrolysis under which the components of the cerebrosides could be obtained in a fair degree of purity and in a quantity approaching the values required by theory. It was found that two operations were required, one for determining the sugar content, and a second for determining the base and acid. In connection with the sugar estimation the older methods particularly needed improvement. During a long period when the older methods of hydrolysis were followed in our laboratory, a yield of glucose approaching the theoretical requirement was obtained only once. A scrutiny of the results published by Thierfelder[2] and his coworkers shows that they also encountered in individual samples of cerebrosides greater sugar variations than one should have expected from the practically constant elementary composition of the substances.

Hence we undertook to determine the destructive influence on galactose of mineral acids in concentrations usually employed for hydrolysis of cerebrosides. The details are given in the tables.

On the basis of these tables the following conditions were selected for the hydrolysis of cerebrosides, aiming at the sugar estimation. 1.0 gm. of cerebrosides and 16 cc. of 3 per cent sulfuric acid were heated with shaking in a sealed tube for 12 hours at 105°. The filtrate from the insoluble cake combined with the wash water was used for the sugar estimation. When larger quantities are employed for hydrolysis the proportions remain the same. Under such conditions approximately 90 per cent of the sugar present in the cerebroside is obtained. We were unable to obtain more accurate results. We also wish to remark that when a small quantity of cerebroside is used the sugar

[2] Loening, H., and Thierfelder, H., *Z. physiol. Chem.*, 1911, lxxiv, 282. Thierfelder, *ibid.*, 1913, lxxxv, 35; 1914, lxxxix, 236. See also Argiris, A., *ibid.*, 1908, lvii, 289.

TABLE I.

Galactose.

I. 3 gm. of galactose dissolved in 100 cc. of 10 per cent HCl + C_2H_5OH, 4:1. Portions of 20 or 10 cc. hydrolyzed for various lengths of time.

	Per cent.	Loss.	Per cent loss.
Original solution	0.546	0	0
8 hrs.	0.380	0.166	30.40
12 "	0.304	0.242	44.40
24 "	0.131	0.415	76.00
48 "	0.022	0.524	96.00

II. 3 gm. of galactose dissolved in 100 cc. of 2 per cent HCl.

	Per cent.	Loss.	Per cent loss.
Original solution	0.511	0	0
2 hrs.	0.511	0	0
4 "	0.4965	0.0145	2.85
8 "	0.4825	0.0285	5.54
24 "	0.4590	0.0520	10.23

III. 3 gm. of galactose dissolved in 100 cc. of 4 per cent H_2SO_4.

	Per cent.	Loss.	Per cent loss.
Original solution	0.5355	0	0
2 hrs.	0.5355	0	0
4 "	0.5105	0.0250	4.66
8 "	0.5070	0.0285	5.34
24 "	0.4820	0.0535	10.00

IV. 1 gm. of galactose and 40 cc. of alcohol containing 7 per cent H_2SO_4 boiled for 4 hours and then hydrolyzed with 3 per cent H_2SO_4 for various lengths of time.

	Sugar.
	gm.
Solution before hydrolysis	0.0000
1 hr. hydrolysis	0.6000
3 " "	0.8925
4 " "	0.6950

TABLE I—*Concluded.*

Phrenosin.

I. 2 gm. of phrenosin boiled with 40 cc. of 10 per cent HCl + 95 per cent alcohol 4: 1 for varying lengths of time.

Amount of phrenosin.	Time of boiling.	Sugar.	
gm.	*hrs.*	*gm.*	*per cent*
2.0000	8	0.205	10.25
1.9946	12	0.084	0.42
1.9974	24	0.031	0.15
2.0004	48	0.000	0.00

II. 3 gm. of phrenosin boiled with absolute alcohol and 40 cc. of 7 per cent H_2SO_4 for 4 hours, and then hydrolyzed with 4.4 per cent H_2SO_4.
Found................11.8 per cent.

III. 3 gm. of phrenosin hydrolyzed with 50 cc. of 3 per cent H_2SO_4 + 5 cc. of alcohol.

	Sugar
hrs.	*per cent*
16	17.2
24	16.3

IV. 1 gm. of phrenosin heated in a sealed tube with 16 cc. of 3 per cent H_2SO_4 at 100° for various lengths of time.

	Sugar.
hrs.	*per cent*
2	6.8
4	15.48
8	16.32
11	17.98
14	18.25
16	17.68
24	16.60

Analyzed according to Rosenheim's method (*Biochem. J.*, 1916, x, 146), 11.56 per cent.

cannot be estimated polarimetrically with a sufficient degree of accuracy.

TABLE II.

Hydrolysis in Sealed Tube for 12 Hours at 105°.

Sample No.	Sugar.
	per cent
60 C	18.8
61 C	18.6
62 C	21.0
272 C	19.2
366 C	18.8
514 C	18.72
Theory.	21.8

For the purpose of estimating the base and the fatty acids the following conditions were found satisfactory: 1 gm. of cerebroside was heated with 3 per cent sulfuric acid in a sealed tube with shaking for 24 hours at 105°. After cooling the reaction product was filtered. The insoluble cake was washed with distilled water on the filter, then pressed between filter paper to remove adhering water. The filter paper with the substance was transferred to boiling methyl alcohol to which a few drops of phenolphthalein were added. This was followed by the addition of a hot saturated solution of barium hydroxide in methyl alcohol until the reaction was strongly alkaline. Acetone was then added as long as a precipitate formed. When all the barium hydroxide and the barium salts are precipitated the pink solution turns colorless. The solution contains the base, and the precipitate consists of the barium salts of the fatty acids. For the purpose of purification the fatty acids were liberated with hydrochloric acid, and redissolved with acetone. This operation was repeated three times. The soaps were suspended in water, decomposed with hydrochloric acid, a little benzene was added, and the flask was placed on a water bath until the benzene was evaporated. On cooling, the acids floated on the surface of the liquid. They were then filtered and dissolved in hot acetone to remove the inorganic salts. The acetone solution on evaporation left a dry residue, ready for analysis. All the mother liquors were added to the original solution containing the base. The combined solu-

tion was evaporated nearly to dryness and extracted with hot acetone. The operation was repeated until the final residue was completely soluble in acetone. All the insoluble residues were added to the soaps. The acetone solution of the bases was evaporated and dried to constant weight.

This method has been tried out for 4 years and has always given results which were as accurate as could be expected for substances of that nature. Table III contains the results of only a few of the analyses carried out by this method.

TABLE III.

Sample No.	Base.	Fatty acid.
	per cent	*per cent*
60 C	32.7	43.0
61 C	31.0	48.6
62 C	30.0	42.8
366 C	35.8	48.52
514 C	32.1	46.7
Theory.	34.5	48.1

The results of the elementary composition of the individual fractions were as follows.

The acids were analyzed directly after removing the acetone, if they were free from inorganic impurities. Otherwise, they were dissolved in ether and freed from impurities by shaking with dilute hydrochloric acid and then with water. The ethereal solution was then freed from ether and the residue analyzed.

60 C 0.1000 gm. substance gave 0.2850 gm. CO_2 and 0.1150 gm. H_2O.
61 C 0.1040 " " " 0.2888 " " " 0.1162 " "
62 C 0.1050 " " " 0.2894 " " " 0.1162 " "
366 C 0.1055 " " " 0.2886 " " " 0.1142 " "
514 C 0.1070 " " " 0.3018 " " " 0.1200 " "

	Calculated for:		Found:				
	$C_{21}H_{43}O_2$:	$C_{25}H_{50}O_3$:	60 C	61 C	62 C	366 C	514 C
C............	78.2	75.33	75.10	75.74	75.16	74.60	77.00
H............	13.2	12.50	12.25	12.50	12.38	12.10	13.03

The base was analyzed in form of the sulfate. In order to obtain this, the fraction was dissolved in alcohol, and an alcoholic solution of sulfuric acid was added until the mixture reacted markedly acid to litmus. The mixture was allowed to stand 15 to 24 hours at 0°C., filtered, and washed, first with acetone and then with ether, and dried under diminished pressure.

60 C 0.1064 gm. substance gave 0.2226 gm. CO_2 and 0.0962 gm. H_2O.
61 C 0.0998 " " " 0.2334 " " " 0.0998 " "
62 C 0.0984 " " " 0.2264 " " " 0.0976 " "
514 C 0.1012 " " " 0.2252 " " " 0.0980 " "

	Calculated for $(C_{17}H_{35}NO_2)_2\ H_2SO_4$:	Found:			
		60 C	61 C	62 C	514 C
C	61.08	60.76	64.42	62.87	61.29
H	10.78	10.00	11.28	11.12	10.95

The analytical figures for the sulfates deviate considerably in some instances from the theory. This however, is not due to the presence of impurities but occurred when an insufficient amount of sulfuric acid was added to form the neutral salt. One or two recrystallizations generally suffice to obtain a pure product. With more experience in the work analytically pure samples are obtainable without recrystallization.

50 gm. of material are a convenient quantity for analysis. Very satisfactory results were obtained when 1.5 gm. were hydrolyzed. When the analysis of the fatty acid fraction obtained by this method shows the presence of both lignoceric and cerebronic acid, and when the identification of each one is desired it is advantageous to resort to a separate hydrolysis for each acid. For the isolation of cerebronic acid, the procedure is as follows.

10 gm. of the cerebrosides and 150 cc. of a 10 per cent hydrochloric acid solution to which 30 cc. of 98 per cent ethyl alcohol are added, are heated for 24 hours in a flask provided with a reflux condensor and a mechanical stirrer. On cooling, a solid cake is formed on the surface of the liquid. This is dissolved in hot methyl alcohol. A solution of barium hydroxide is then added and the soaps are purified exactly in the manner described above.

The acid obtained in this manner generally analyzes as follows:

A sample of cerebroside (94 C) with $[\alpha]_D^{20} = 0.00°$:
0.1144 gm. substance gave 0.3176 gm. CO_2 and 0.1257 gm. H_2O.
C = 75.71; H = 12.30.
A sample of cerebroside (221 B) with $[\alpha]_D^{20} = -2.0°$:
0.1166 gm. substance gave 0.3248 gm. CO_2 and 0.1317 gm. H_2O.
C = 75.85; H = 12.64.

From the optical rotation of the substances it is evident that they contained cerasin. On the other hand samples of the same nature yield pure lignoceric acid when decomposed by means of alcohol containing sulfuric acid. The details are as follows.

10.0 gm. of cerebroside are taken up in 100 cc. of 99.5 per cent alcohol containing 10.0 gm. of sulfuric acid and heated in a boiling water bath for 8 hours. The solution is allowed to cool over night at 25°C. Ethyl lignocerate settles out in the form of bright scales. These are removed by filtration. The precipitate may be dissolved in ether, the solution dried over potassium carbonate, filtered, and analyzed in the form of the ester. It also may be converted into the free acid.

Treated in this manner a sample (24 B) with $[\alpha]_D^{20} = 0.00°$ yielded an acid of the following composition:

0.1183 gm. substance gave 0.3388 gm. CO_2 and 0.1139 gm. H_2O.
C = 78.10; H = 13.15.

CEREBROSIDES.

IV. CERASIN.

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Thudichum[1] was the first to assume the existence of more than one cerebroside in brain tissue. By fractionation from alcohol he succeeded in isolating from the crude cerebroside mixture two substances. One he considered an individual glucoside, which he named phrenosin. The other substance he regarded as a second cerebroside, cerasin, still containing some phrenosin. Apparently for lack of material, Thudichum did not accomplish the task of preparing a pure sample of cerasin, nor did he furnish any information regarding the chemical differences of the two cerebrosides.

Subsequent workers have rediscovered phrenosin[2] and, in a general way, have substantiated Thudichum's claim for its individuality.

Regarding the second cerebroside, cerasin, no essential progress was made for many years. The substances, phrenosin and cerasin, were distinguished by their solubilities and their physical appearance. However, in 1913, a chemical distinction between phrenosin and cerasin was twice discovered, nearly simultaneously. It was found that from the so called cerasin, a fatty acid of the composition, $C_{24}H_{48}O_2$, could be isolated, whereas pure phrenosin contained only cerebronic acid, $C_{25}H_{50}O_3$. In this respect it is worthy of note that Thudichum foresaw the possibility of the two cerebrosides differing only by the nature of their

[1] Thudichum, J. L. W., Physiological Chemistry of the Brain, London, 1884.

[2] Loening, H., and Thierfelder, H., *Z. physiol. Chem.*, 1911, lxxiv, 282; 1912, lxxvii, 202. Thierfelder, *Z. physiol. Chem.*, 1913, lxxxv, 35; 1914, lxxxix, 236.

fatty acids. The new acid was recognized independently by Levene[3] and by Rosenheim[4] as lignoceric acid. There still seems, however, to exist a lack of convincing evidence that cerasin has ever been obtained free from phrenosin. It is true, on the basis of a new test applied to the study of cerebrosides, namely, the selenite plate test, Rosenheim claimed to have isolated pure cerasin. However, comparing the optical activity of the various samples obtained by Rosenheim with those prepared by us, we feel certain that our material was of the same degree of purity as that of the previous author. The samples of Rosenheim varied between $[\alpha]_D = -2.50°$ and $-3.71°$, whereas the $[\alpha]_D$ of our samples varied between $-2.25°$ and $-3.45°$.

The first samples of cerasin of that nature were obtained by us in 1913. The results were not published at that time for the reason that we hoped to obtain purer material by means of the older methods of fractional crystallization, or fractional extraction by means of organic solvents. We soon became convinced, however, that the results of the operations were variable, even when the conditions of purification seemed constant. Evidently some unknown factors played a part and since the factors remained unknown, they could not be controlled. Hence the method of fractionation was abandoned and a search was made for such derivatives of phrenosin and cerasin, which would possess properties suitable for the separation of one from the other.

With this aim in view the properties of the acetyl, benzoyl, cinnamoyl, and *p*-nitrobenzoyl derivatives were investigated. Benzoylation was selected as the simplest and best method for the separation of the two compounds. A convenient way of benzoylation was found in the use of pyridine as a solvent, a method first introduced by Einhorn. The saponification of the benzoyl derivative is best accomplished by means of sodium methylate. These are the two essential points of the process, the details of which are given in the experimental part.

It should be noted here that Rosenheim[5] mentioned the possibility of accomplishing the separation of the two cerebrosides

[3] Levene, P. A., *J. Biol. Chem.*, 1913, xv, 359.

[4] Rosenheim, O., *Tr. Int. Cong. Med.*, 1913, ii, 626; *Biochem. J.*, 1916, x, 142.

[5] Rosenheim, *Biochem. J.*, 1914, viii, 110.

through benzoylation. Thierfelder[6] later suggested the use of the acetyl derivatives.

It must, however, be borne in mind that a satisfactory result is obtained only when the starting material contains a moderate proportion of phrenosin; namely, a mixture with $[\alpha]_D = 0.0°$ in pyridine. Hence, the complete process is composed of an initial step consisting of fractional precipitation or fractional extraction, and a second step, consisting of separation by means of benzoylation. As a result of this second step a product is obtained with $[\alpha]_D = -2.50$ to $-3.50°$.

This levorotation is nearly that obtained on previous occasions by Rosenheim in 1916 and by us in 1913. As in 1913, we are convinced now also that this material is not yet free from phrenosin. This view is based on the fact that, on hydrolysis with aqueous sulfuric acid under conditions described by Levene and Meyer,[7] cerasin, with the highest levorotation, yields a mixture of fatty acids containing about 77.0 per cent carbon. Furthermore, on hydrolysis in a solution of 10 per cent hydrochloric acid, containing 15 per cent alcohol, one obtains either pure cerebronic acid, or a mixture of cerebronic and lignoceric acids, whereas, by using alcohol containing 8 to 10 per cent sulfuric acid (by weight), one obtains pure lignoceric acid. Hence, it is evident that one may be led into an error if he bases his conclusion on the latter mode of cleavage (alcoholysis). The find of Rosenheim may be explained by the fact that he employed this method of hydrolysis.

Thus, a complete separation of cerasin from phrenosin has not as yet been accomplished. The present results are reported at this time because of lack of confidence in a speedy solution of the problem. For the present we are engaged in the preparation of large quantities of material with a rotation of approximately $-3.0°$.

In a way the topic discussed here may appear to possess a merely academic interest, since the existence of the two cerebrosides is quite certain. The only alternative hypothesis would be the existence of one simple cerebroside, phrenosin, while cerasin might be a dicerebroside. This view is contradicted by the molecular

[6] Thierfelder, *Z. physiol. Chem.*, 1914, lxxxix, 248.

[7] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1917, xxxi, 627.

weight determinations by Kossel and Freytag,[8] who found a mean value of 986, using glacial acetic acid as the solvent, and by Rosenheim,[9] who found a mean of 763, using Barger's microscopic method, based on vapor pressure, with pyridine as the solvent. However, the final and absolute proof of the existence of a substance is its isolation.

EXPERIMENTAL.

Preparation of the Mixture of Cerebrosides.

The crude cerebrosides used in this work were prepared essentially by the method of Rosenheim.[10] The principal feature of this method consists in dissolving the "white matter" (mixture of cerebrosides and sphingomyelin) in pyridine. The difference in the procedure was that the "white matter" was heated in commercial pyridine, until the solution was complete. This solution was then allowed to stand over night at room temperature. The filtrate contained the cerebrosides. This was concentrated to a small volume and poured into alcohol. The precipitate thus obtained was dissolved in hot 90 per cent alcohol, filtered from insoluble oily material, and again allowed to cool. The precipitate was repeatedly extracted with ether. The final insoluble product was composed of the mixed cerebrosides.

Separation into Phrenosin and Cerasin Fractions.

The separation into phrenosin and cerasin fractions was accomplished by means of acetone. Two fractions were obtained, one practically insoluble in boiling acetone, the other soluble. Each of these fractions was a mixture of two cerebrosides. However, the first (insoluble) contained a very high proportion of phrenosin while in the second (soluble) cerasin was predominating.

The procedure used was as follows: 100 gm. of the cerebrosides were dissolved in 50 cc. of boiling 95 per cent ethyl alcohol and to this solution 3 liters of boiling acetone were added. A precipitate formed immediately. This was removed by filtering with suction,

[8] Kossel, A., and Freytag, F., *Z. physiol. Chem.*, 1893, xvii, 445.

[9] Rosenheim, *Biochem. J.*, 1916, x, 153.

[10] Rosenheim, *Biochem. J.*, 1914, viii, 110; 1916, x, 142.

care being taken to warm the funnel before filtration. The insoluble part yields practically pure phrenosin when redissolved in boiling alcohol and then allowed to crystallize in a thermostat at 42°C.

The acetone-solution fraction was concentrated to a small volume and the cerebrosides were allowed to separate out. The material obtained in this manner was further fractionated from methyl alcohol. For this purpose it was dissolved in methyl alcohol and then allowed to stand at 40°. This gave the fraction I a_1. The filtrate from I a_1 was then allowed to cool to room temperature when a second precipitate settled out. This was designated I a_2. On cooling to 0°C. a third fraction, I a_3, was obtained.

The further purification of each of these fractions was laborious, various modifications of Rosenheim's original method being tried, and the results were variable. These fractions have no interest since the introduction of the benzoylation process. In order to obtain material for this process, the crude cerebrosides may be dissolved in a hot solution of one part ethyl alcohol and three parts chloroform (by volume). On standing at room temperature a precipitate forms, which is removed by filtration. The mother liquor is concentrated and either allowed to crystallize or is poured into acetone. Usually this material has a rotation of about 0.0° (in pyridine). If it is higher (+0.02 to +0.04°) the operation is repeated. In some cases it was found that the mother liquor of the crystallization of the crude cerebrosides from ten parts of ethyl alcohol also gave a product with a rotation of $[\alpha]_D = 0.0°$.

Further Separation of Cerasin from Phrenosin by Benzoylation.

As mentioned above, Rosenheim[5] first suggested the possibility of a separation of phrenosin and cerasin by means of the benzoyl derivatives. We have also examined the acetyl derivatives as a method of separation but find it unsatisfactory, especially when compared with the benzoyl method.

The acetyl derivative of the material with a rotation of about −0.02° was prepared as described below and separated into two fractions, soluble and insoluble in methyl alcohol at 0°. The two fractions were then saponified with sodium methylate, and gave products with $[\alpha]_D$ of − 0.02° and − 0.02°. This indicates that no separation has been effected.

The benzoyl derivative was prepared in pyridine as described on page 644. The product, after removing the ether on the water bath, is taken up in methyl alcohol and cooled to 0° over night. The supernatant liquid is decanted, concentrated to a small volume, poured into a solution of sodium methylate in methyl alcohol, and the mixture heated 2 hours. Usually the cerebroside separates out during the heating. After cooling, the precipitate is filtered off, washed thoroughly with methyl alcohol and then with acetone, and crystallized from methyl alcohol until ash-free. It is usually necessary to decolorize the solution with animal charcoal in order to obtain a colorless product. Such material showed values of $[\alpha]_D$ varying from -2.2 to $-3.2°$ in pyridine.

$$[\alpha]_D^{20} = \frac{7.4810 \times -0.08°}{0.4180 \times 0.5} = -2.86°$$

$$[\alpha]_D^{20} = \frac{7.9450 \times -0.07°}{0.5028 \times 0.5} = -2.25°$$

$$[\alpha]_D^{20} = \frac{6.1186 \times -0.08°}{0.306 \times 0.5} = -3.2°$$

Since the first purification through the benzoyl derivative was so successful, it was thought that the repetition of the process would yield a cerasin with a still higher rotation. A product with $[\alpha]_D^{20} = -2.86°$ was therefore benzoylated and the methyl alcohol mother liquor saponified as above. The cerebroside, twice crystallized from methyl alcohol, showed a value for $[\alpha]_D^{20}$ of $-2.98°$.

$$[\alpha]_D^{20} = \frac{5.101 \times -0.10°}{0.3440 \times 0.5} = -2.98°$$

This indicates little or no purification. No better results were obtained with the acetyl derivative. The material before acetylation had a value of $[\alpha]_D$ of $-2.30°$, and the product after saponification of the acetyl derivative showed $[\alpha]_D = -2.34°$.

Hydrolysis of Cerasin Obtained by Fractionation.

The fraction most soluble in methyl alcohol was further fractionated out of pyridine and chloroform, which gave a product with a rotation of $-2.24°$.

$$[\alpha]_D^{20} = \frac{5.281 \times -0.17^\circ}{0.400 \times 1} = -2.24^\circ$$

30 gm. of this material were hydrolyzed with 450 cc. of 10 per cent hydrochloric acid and 75 cc. of 95 per cent alcohol. The acids obtained from this hydrolysis, worked up in the usual way, had the following composition:

0.1167 gm. substance gave 0.3293 gm. CO_2 and 0.1324 gm. H_2O.

	Calculated for $C_{24}H_{48}O_2$:	Calculated for C_{2}[illegible]$H_{50}O_3$:	Found:
C	78.20	75.33	76.95
H	13.16	12.50	12.70

The fraction I a_2 (page 639) was extracted with dilute acetone (one part water to ten parts acetone). The insoluble part had a rotation of -3.45°.

$$[\alpha]_D^{20} = \frac{5.2834 \times -0.28^\circ}{0.400 \times 1} = -3.45^\circ$$

20 gm. of this material were hydrolyzed by boiling for 6 hours with 150 cc. of 98 per cent alcohol containing 7.5 cc. of sulfuric acid. On standing over night at room temperature, scales of ethyl lignocerate separated out.

0.1129 gm. substance gave 0.3240 gm. CO_2 and 0.1318 gm. H_2O.

	Calculated for $C_{26}H_{52}O_2$:	Found:
C	78.8	78.26
H	13.10	13.16

15 gm. of the same sample were then hydrolyzed with 150 cc. of 10 per cent hydrochloric acid and 15 cc. of 95 per cent alcohol. The fatty acids were obtained through the barium salts. The acids were then transferred into the ethyl ester by boiling in a solution of 100 cc. of 98 per cent alcohol containing 5 gm. sulfuric acid for 6 hours. On standing, scales separated out, with the following composition.

0.1150 gm. substance gave 0.3264 gm. CO_2 and 0.1306 gm. H_2O.

	Calculated for $C_{26}H_{52}O_2$:	Found:
C	78.80	77.40
H	13.10	12.71

Hydrolysis of Cerasin Obtained by the Benzoylation Process.

3 gm. of the material with $[\alpha]_D^{20} = -3.25°$ were heated in a sealed tube with 50 cc. of 3 per cent sulfuric acid at 100–105° for 18 hours. The acids were isolated through the barium salts and had the following composition.

0.1044 gm. substance gave 0.2940 gm. CO_2 and 0.1152 gm. H_2O.

	Calculated for $C_{24}H_{48}O_2$:	Calculated for $C_{25}H_{50}O_3$:	Found:
C	78.20	75.33	76.80
H	13.16	12.50	12.35

Acetyl Phrenosin.

Acetyl phrenosin was first prepared by Thierfelder.[6] 20 gm. of phrenosin, 20 gm. of fused sodium acetate, and 200 cc. of acetic anhydride were heated to gentle boiling for ½ hour. The excess of acetic anhydride was removed by distillation in vacuum and the semisolid residue dissolved in ether and water. The ether solution of the acetyl derivative was washed with water, with dilute alkali, and again with water, dried over sodium sulfate, and the ether concentrated on the water bath. The residue was taken up in hot dry methyl alcohol, in which it is very soluble, and from which it separates in a granular condition upon cooling. After the second crystallization from methyl alcohol, the yield was 14 gm. of a product with $[\alpha]_D = -10.4°$. The third crystallization gave a product with $[\alpha]_D = -11.07°$, which was not changed on further purification.

Acetyl phrenosin melts somewhat unsharply at 41–43°. Thierfelder gives the melting point as 39–41° and the rotation as $-3°$. Our product, dissolved in a mixture of equal parts of chloroform and methyl alcohol (by volume) showed the following rotation:

$$[\alpha]_D^{20} = \frac{7.0708 \times -0.39°}{0.4980 \times 0.5} = -11.07°$$

$$[\alpha]_D^{20} = \frac{6.5570 \times -0.43°}{0.5066 \times 0.5} = -11.08°$$

0.1052 gm. substance gave 0.2568 gm. CO_2 and 0.0892 gm. H_2O.

0.5000 gm. substance neutralized 4.35 cc. 0.1 N HCl.

0.500 gm. substance, used for an acetyl determination, required 28 cc. 0.1 N HCl, and in a second experiment, 28.2 cc.

0.673 gm. substance, in 22.56 gm. chloroform produced a rise in boiling point of 0.108°.

0.943 gm. substance, in 22.56 gm. chloroform, produced a rise in boiling point of 0.158°.

	Calculated for hexacetylphrenosin $C_{60}H_{105}NO_{15}$:	Found:	
C	66.68		66.57
H	9.80		9.49
N	1.29		1.22
Ac	23.8	24.08	24.24
Mol. wt.	1079	1003	960

One experiment was made to determine whether the same derivative could be prepared by the use of acetyl chloride. 20 gm. of phrenosin were dissolved in 150 cc. of pyridine, the solution was cooled to 0°C., 16 cc. of acetyl chloride were added in portions, and the mixture was allowed to stand 2 days at 0°C. The pyridine hydrochloride was filtered off, the solution washed with water, dilute hydrochloric acid, and then with water, dried with sodium sulfate, and concentrated. The residue was crystallized repeatedly from methyl alcohol. After the fourth crystallization, it had a rotation of −8.41°, and melted at about 40°.

$$[\alpha]_D^{20} = \frac{6.1218 \times -0.33^\circ}{0.4794 \times 0.5} = -8.41^\circ$$

The analysis indicates that it is probably a mixture of acetyl derivatives. It was not purified further.

0.1008 gm. substance gave 0.2484 gm. CO_2 and 0.0896 gm. H_2O.

	Calculated for Hexacetyl-phrenosin $C_{60}H_{205}NO_{15}$:	Calculated for Triacetyl-phrenosin $C_{54}H_{99}NO_{12}$:	Found:
C	66.68	67.94	67.22
H	9.80	10.46	9.96

Acetyl Cerasin.

20 gm. of a cerasin with $[\alpha]_D^{20} = -2.8^\circ$, 20 gm. of sodium acetate, and 200 cc. of acetic anhydride were boiled under a reflux for ½

hour. The reaction product was worked up as described above, and the product then twice crystallized from methyl alcohol. This material did not show a change in rotation upon further purification from methyl alcohol. It is slightly more insoluble in methyl alcohol than is acetyl phrenosin, as stated by Thierfelder.[6] Acetyl cerasin melts at 54–56°, and in a mixture of equal parts of chloroform and methyl alcohol gave the rotation:

$$[\alpha]_D^{20} = \frac{5.9310 \times -0.60^\circ}{0.4324 \times 0.5} = -16.46^\circ$$

0.1035 gm. substance gave 0.2576 gm. CO_2 and 0.0932 gm. H_2O.
0.500 gm. substance neutralized 4.4 cc. 0.1 N HCl.

	Calculated for pentacetylcerasin $C_{57}H_{101}NO_{13}$:	Found:
C	67.87	67.75
H	10.10	10.00
N	1.40	1.23

As stated on page 639, the acetyl derivatives are not a suitable means of separating phrenosin and cerasin.

Benzoylphrenosin.

10 gm. of practically pure phrenosin were dissolved in 100 cc. of pyridine, cooled to room temperature, and treated with 12 cc. of benzoyl chloride. The reaction mixture was cooled under the water tap and then allowed to stand at 0°C. over night. The next day the pyridine hydrochloride was filtered off, the solution concentrated in vacuum at 50°C., and the resulting oily liquid poured, with stirring, into a large volume of about 2 per cent sodium hydroxide solution. The benzoylphrenosin separated as an oil on the sides and bottom of the dish. After washing with water and hydrochloric acid, the material was taken up in ether and dried with sodium sulfate. An alternative procedure is to dissolve the pyridine containing oily material in ether, then shake with dilute acid and alkali, then with water, and dry this solution. After removal of the ether the product is taken up in hot methyl alcohol. From a concentrated solution, the benzoyl derivative separates as an oil, which solidifies on cooling. From a sufficiently dilute solution it separates as colorless nearly crystal-

line material, which is easily filtered. It may also be obtained in a granular form from ethyl alcohol. It is easily soluble in acetone, benzene, petroleum ether, chloroform, acetic acid, and pyridine.

In an experiment in which 80 gm. of phrenosin were used, the benzoyl derivative was purified by crystallization from methyl alcohol, then from acetone (in which about 50 per cent of the material was lost), and then from methyl alcohol until the rotation was constant. The melting point as observed on this material was fairly sharp at 65–66°.

In methyl alcohol and chloroform the rotation is:

$$\text{I.}\quad \left[\alpha\right]_{\mathrm{D}}^{20} = \frac{7.1484 \times +0.75^\circ}{0.5062 \times 0.5} = +21.1^\circ$$

$$\text{II.}\quad \left[\alpha\right]_{\mathrm{D}}^{20} = \frac{6.1518 \times +0.92^\circ}{0.5320 \times 0.5} = +21.27^\circ$$

$$\text{III.}\quad \left[\alpha\right]_{\mathrm{D}}^{20} = \frac{6.1060 \times +0.91^\circ}{0.5240 \times 0.5} = +21.20^\circ$$

Sample I was obtained from material which contained at least 75 per cent cerasin. Samples II and III were obtained from material considered nearly pure phrenosin. The analysis and molecular weight indicate that this material of constant rotation was probably a tribenzoylphrenosin.

0.1004 gm. substance gave 0.2704 gm. CO_2 and 0.0792 gm. H_2O.
0.1028 " " " 0.2784 " " " 0.0866 " "
0.1000 " " " 0.2707 " " " 0.0776 " "
0.5000 " " neutralized 4.40 cc. 0.1 N HCl.
0.5000 " " " 4.10 " 0.1 "
0.500 " " used for a benzoyl determination required 11.6 cc. 0.1 N NaOH, and in a second experiment 11.9 cc.
0.255 " " in 23.21 gm. chloroform raised the boiling point 0.026°.
0.682 " " as above, raised the boiling point 0.108°.
1.008 " " " " " " " " 0.160°.

	Calculated for tribenzoylphrenosin $C_{69}H_{105}NO_{12}$:	Found:		
C	72.65	73.45	73.89	73.81
H	9.28	8.83	9.43	8.69
N	1.23		1.23	1.12
C_6H_5CO	25.32		24.36	24.99
Mol. wt.	1,139	1,037	1,066	1,092
Average			1,065	

$C_{69}H_{10}$

An attempt was then made to prepare a hexabenzoyl derivative. 40 gm. of the above mentioned material were dissolved in 300 cc. of pyridine and treated with 400 cc. of benzoyl chloride. After working up as usual, the product was taken up in methyl alcohol. An oil formed which would not solidify even after standing several weeks. Since the material with constant rotation served our need the experiment was discarded.

Phrenosin from Benzoÿlphrenosin.

About 10 gm. of sodium were dissolved in 300 cc. of methyl alcohol and 20 gm. of benzoylphrenosin added with shaking (the benzoylphrenosin may also be added in acetone solution). A white precipitate gradually forms upon boiling (immediately, if an acetone solution is used). After boiling for 2 hours, the reaction product is cooled in the ice box, the precipitate filtered off, recrystallized from methyl alcohol, then from glacial acetic acid, and finally from a mixture of equal parts of methyl and ethyl alcohols.

The phrenosin crystallizes practically quantitatively for upon concentration of the mother liquor only sodium benzoate was obtained. The rotation of this material in pyridine corresponds well with that found for the phrenosin obtained by fractionation of the top fraction of the cerebroside mixture. Rosenheim gives +3.78° and +3.70°.

In pyridine the rotation was:

$$\left[\alpha\right]_{D}^{20} = \frac{+8.0000 \times 0.12^{\circ}}{0.5232 \times 0.5} = +3.67^{\circ}$$

Cinnamoylphrenosin.

10 gm. of phrenosin were dissolved in 100 cc. of pyridine and 15 gm. of cinnamoyl chloride added. After standing over night at 0°, the filtered solution was concentrated and worked up as described above. The resulting product was purified by crystallization from methyl or ethyl alcohol, or from acetone. Twice recrystallized from methyl alcohol, and once from acetone, the substance analyzed as a tricinnamoylphrenosin. Tricinnamoylphrenosin is slightly less soluble in organic solvents than the benzoyl derivative. It melts at 69–70°.

$$[\alpha]_{D}^{20} = \frac{7.2060 \times 0.75°}{0.4976 \times 85} = +21.72°$$

0.1028 gm. substance gave 0.2718 gm. CO_2 and 0.0868 gm. H_2O.
0.1016 " " " 0.2732 " " " 0.0870 " "
0.500 " " neutralized 4.3 cc. 0.1 N HCl.

	Calculated for tricinnamoylphrenosin $C_{75}H_{111}NO_{12}$:	Found:	
C	73.89	73.22	73.33
H	9.18	9.45	9.58
N	1.15	1.20	

p-Nitrobenzoylphrenosin.

20 gm. of phrenosin, dissolved in 200 cc. of pyridine, were treated with 32 gm. of *p*-nitrobenzoyl chloride, and the mixture was allowed to stand 24 hours at 0°. The reaction product was worked up as usual. The residue from the ether was extracted with hot acetone, the acetone removed on the steam bath, and the product extracted with boiling methyl alcohol. On cooling, the larger part of the nitrobenzoate separated as an oil. This was again extracted with boiling methyl alcohol, and the resulting cake twice crystallized from a large volume of methyl alcohol. *p*-Nitrobenzoylphrenosin is very soluble in acetone, and melts at 94–96°. Analysis indicates that tri-*p*-nitrobenzoylphrenosin is formed. The rotation in chloroform and methyl alcohol was:

$$[\alpha]_{D}^{20} = \frac{8.6510 \times 0.35°}{0.4970 \times 0.50} = +12.18°$$

0.1058 gm. substance gave 0.2542 gm. CO_2 and 0.0810 gm. H_2O.
0.1000 " " " 4.00 cc. N gas at 22°C. and 767 mm.

	Calculated for $C_{69}H_{102}N_4O_{18}$:	Found:
C	65.00	65.52
H	8.06	8.57
N	4.40	4.67

CEREBROSIDES.

V. CEREBROSIDES OF THE KIDNEY, LIVER, AND EGG YOLK.

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With the progress of our knowledge of the lipoids of the various organs of the animal body there comes a conviction that there does not exist much variation in the chemical composition of the individual lipoids with the variation of the organs or tissues from which they originate. Up to the present this has been found true in regard to cephalin[1] and sphingomyelin.[2] The present note contains data regarding the cerebrosides obtained from the same sources. The cerebrosides are found in organs other than the nervous system in very small proportions. Because of this the purification is associated with many difficulties. Furthermore, it is nearly impossible to obtain sufficiently large quantities of material for an exhaustive analysis.

For the present, one is satisfied to be able to show the presence of cerebrosides in several tissues—in the egg yolk, in the liver, and in the kidney—and to be able to state that the composition of these cerebrosides seems to be identical with that of the cerebrosides obtained from the nerve tissue. They contain the same sugar, galactose, the same base, sphingosine, and the same fatty acids, lignoceric and cerebronic.

It is not certain whether the two cerebrosides, phrenosin and cerasin, occur in the same proportions in nerve tissue and in the various other organs. The same remark applies regarding every other individual lipoid. It is possible that the proportions do vary with the variation of the organ.

Historically it must be mentioned that the first evidence of existence of cerebrosides in other organs than nervous tissue was

[1] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1916, xxiv, 111.

[2] Levene, *J. Biol. Chem.*, 1916, xxiv, 69.

furnished by Hoppe-Seyler,[3] and later by Kossel and Freytag,[4] who discovered the presence of cerebrosides in pus cells. Recently Rosenheim and MacLean[5] showed, by the isolation of lignoceric acid and sphingosine from the so called carnaubon of Dunham,[6] that the kidney probably contained cerebrosides, but these were not isolated in a pure state.

The following table gives the composition and optical activity of the various cerebrosides thus far studied.

	Source.	C	H	N	$[\alpha]_D^{20}$
Phrenosin....................	Brain.	69.51	11.37	1.69	9.6* (3.6)**
Cerasin......................	"	69.53	11.70	1.68	−3.25**
Cerebrosides.................	Kidney.	69.60	11.30	1.68	+14.00*
"	Liver.	68.36	11.23	1.80	
"	Egg yolk.	69.32	11.19	1.89	+4.40*
Theory for phrenosin.......		69.65	11.23	1.70	
" " cerasin..........		71.20	11.33	1.73	

* In chloroform and methyl alcohol.

** In pyridine.

EXPERIMENTAL.

Kidney Cerebrosides.

The preparation of the cerebrosides from the kidney was accomplished by following the general plan outlined in the previous communication.[7] However, in place of pure cerebrosides which are obtained from nerve tissue by this process, the material prepared from the crude kidney "white matter" contained a considerable proportion of neutral fat, the removal of which was found very troublesome. A great aid for the purpose of purification of this material was found in the use of methyl ethyl ketone. By repeated crystallization from this reagent of the crude cerebrosides a substance was finally obtained which had all the properties

[3] Hoppe-Seyler, F., *Med.-Chem. Untersuch.*, 1866–71, 486.

[4] Kossel, A., and Freytag, F., *Z. physiol. Chem.*, 1893, xvii, 452.

[5] Rosenheim, O., and MacLean, H., *Biochem. J.*, 1915, ix, 103.

[6] Dunham, E. K., *J. Biol. Chem.*, 1908, iv, 297. Dunham, E. K., and Jacobson, C. A., *Z. physiol. Chem.*, 1910, lxiv, 302.

[7] Levene and West, *J. Biol. Chem.*, 1917, xxxi, 635.

of the mixed cerebrosides. This material gave with orcin the typical test for galactose, and on hydrolysis yielded sphingosine and the typical fatty acid mixture.

0.0984 gm. substance gave 0.2456 gm. CO_2 and 0.0972 gm. H_2O.
0.500 " " neutralized 6 cc. 0.1 N HCl.

	Calculated for phrenosin:	Found:
C	69.65	69.60
H	11.24	11.30
N	1.70	1.68

The optical rotation of the substance, in a mixture of equal parts of chloroform and methyl alcohol (by volume) was:

$$[\alpha]_D^{20} = \frac{9.5312 \times 0.26°}{0.3550 \times 0.5} = +14.00°$$

Hydrolysis of the Mixed Cerebrosides.

1.5 gm. of the mixed cerebrosides were heated with 75 cc. of 3 per cent sulfuric acid for 24 hours in a sealed tube at 105°. The base and acids were separated and prepared for analysis as described in a previous article.

The acids had the following composition:

0.1012 gm. substance gave 0.2978 gm. CO_2 and 0.1098 gm. H_2O.

	Calculated for $C_{25}H_{50}O_3$:	Calculated for $C_{24}H_{48}O_2$:	Found:
C	75.33	78.20	75.40
H	12.50	13.20	12.18

Thus the acid was apparently nearly pure cerebronic acid, $C_{25}H_{50}O_3$.

The base was transformed into the sulfate and gave the following figures on analysis.

0.0990 gm. of substance gave 0.2264 gm. CO_2 and 0.0910 gm. H_2O.

	Calculated for $(C_{17}H_{35}NO_2)_2H_2SO_4$:	Found:
C	61.08	62.36
H	10.78	10.29

Crude sphingosine sulfate, previous to crystallization, very frequently gives analytical data as in the present experiment. There is little doubt that the base of the kidney cerebrosides is sphingosine.

Liver Cerebrosides.

Desiccated and pulverized liver tissue was allowed to stand over night with 95 per cent alcohol and then filtered. The residue was repeatedly extracted with boiling alcohol, each extraction lasting about ½ hour. The combined extracts, on standing in the refrigerator, at 0°, gave a dark, nearly black deposit, which corresponds to the "white matter" of the brain extracts. This deposit was extracted in the cold progressively with acetone, alcohol, and ether. The still dark but quite dry mass was fractionated into two parts by dissolving it in hot pyridine and allowing it to cool to room temperature. The mother liquor containing the cerebrosides was concentrated and poured into acetone. The precipitate thus obtained was still very dark. For further purification it was boiled with hot alcohol; a small part remained insoluble. The solution was decanted and a concentrated solution of barium hydroxide was added as long as a precipitate formed. This mixture was allowed to stand in the ice box, and the precipitate which formed was repeatedly extracted with boiling alcohol. The extracts, upon cooling to 0°, gave a precipitate which had the appearance and properties of the brain cerebrosides. This product was then repeatedly extracted with ether, when analysis showed that it was still contaminated with large amounts of neutral fat. The purification was then continued by extraction with ether and by crystallization from acetic acid. The product, however, persisted in containing neutral fat. Finally, the product was dissolved in hot methyl ethyl ketone, from which it settled out on cooling. This was repeated three times, the product then having the composition of cerebrin. Because of the great losses connected with the purification it was not possible to obtain sufficient material for hydrolysis. The test for galactose was positive and there is little doubt that we are dealing with a characteristic cerebroside mixture.

0.1006 gm. substance gave 0.2522 gm. CO_2 and 0.1010 gm. H_2O.

	Calculated for phrenosin:	Found:
C	69.65	68.36
H	11.24	11.23

Egg Cerebrosides.

Egg yolk (dried commercial egg yolk was used in all the work) was thoroughly extracted with acetone at room temperature, to remove egg oil. The material was then extracted with boiling alcohol, repeatedly, as in the former preparations. The combined alcoholic extracts were concentrated to a small volume, and repeatedly treated with acetone, to complete the removal of the egg oil. The acetone-insoluble fraction (lecithin, cephalin, cerebrosides, and saturated phosphatides) was extracted with ether. A small part did not go into solution. The ether suspension was centrifuged, the insoluble material suspended in acetone, filtered off, and again extracted with ether. The insoluble material then corresponded to "white matter" previously mentioned, and is the material analyzed by Stern and Thierfelder[8] and considered impure diaminomonophosphatide. It was later given the name *albin* by Bing and Ellermann.[9]

This was fractionated out of pyridine as described above. The cerebroside fraction was crystallized out of glacial acetic acid and the neutral fat removed by repeatedly extracting with acetone at 50°, and finally by crystallization from methyl ethyl ketone. This material possessed all the physical properties of the mixed cerebrosides, and gave the galactose test with orcin.

0.1020 gm. substance gave 0.2476 gm. CO_2 and 0.0974 gm. H_2O.
0.5000 " " neutralized 6.75 cc. 0.1 N HCl.

	Calculated for phrenosin:	Found:
C	69.65	69.32
H	11.24	11.19
N	1.70	1.89

The optical activity of the material in a mixture of methyl alcohol and chloroform was:

$$[\alpha]_D^{20} = \frac{12.4370 \times 0.11^\circ}{0.3062 \times 0.5} = +4.40^\circ$$

[8] Stern, M., and Thierfelder, H., *Z. physiol. Chem.*, 1907, liii, 370.

[9] Bing, H. J., and Ellermann, V., *Biochem. Z.*, 1912, xlii, 289.

Hydrolysis of Egg Cerebrosides.

1.5 gm. of the substance were heated in a sealed tube with 75 cc. of 3 per cent sulfuric acid for 24 hours at 105°.

The acids and bases were prepared in the manner described above.

0.1000 gm. substance gave 0.2708 gm. CO_2 and 0.1070 gm. H_2O.

	Calculated for $C_{25}H_{50}O_3$:	Found:
C	75.33	74.52
H	12.50	12.09

The base was analyzed as the sulfate.

0.0905 gm. substance gave 0.2024 gm. CO_2 and 0.0810 gm. H_2O.

	Calculated for $(C_{17}H_{35}NO_2)_2H_2SO_4$:	Found:
C	61.08	60.99
H	10.78	10.01

INDEX.